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(With 5 Diagrams)

THE VARIATION IN COMPOSITION DURING LACTATION

IN a previous communication some analytical data concerning the composition of mare's milk were recorded (Linton(1)). The milks analysed included 104 samples obtained from mares during the post-colostral period and thirty-eight drawn during the first few days of lactation while the milk was still of a colostral nature. The period covered was from the day of parturition to the 270th day *post partum*, but the majority of the post-colostral samples were taken during the first month *post partum*. Milk was obtained from Clydesdale, Shire, Thoroughbred, Hunter and Pony mares. Analysis of the colostrum-free milk gave the following results:

	Total solids	Protein	Fat	Lactose	Ash
Average (104)	10·96	2·69	1·59	6·14	0·51
Maximum	18·74	7·60	7·88	8·78	0·95
Minimum	5·93	0·55	0·09	1·65	0·28

From the percentages recorded by previous investigators these average figures were computed:

	Total solids	Protein	Fat	Lactose	Ash
Mean	9·78	2·18	1·59	5·93	0·38
Maximum	11·20	3·34	2·50	7·26	1·20
Minimum	7·47	1·33	0·12	3·20	0·26

They represent the results obtained from the analysis of the milk from Russian, French and German mares, and also from a few British mares.

When reviewing these analytical data an attempt was made to give some average figures which might be regarded as representative of mare's normal milk. It was noted that the average of the percentages which had been published previously and the average composition of the 104 analyses made by the writer were in fairly close agreement, and it was therefore suggested that for the milk of British mares the average composition might be assumed to be:

Total solids	Protein	Fat	Lactose	Ash
10·96	2·69	1·59	6·14	0·51

Nevertheless, considerable variations in the percentages of the milk constituents, and especially of the ash, were observed to occur. It was also noted that the mineral content of the milk secreted by British mares, especially that secreted by the mares of the heavier breeds, Clydesdales and Shires, was on the whole greater than that found in the milk of Continental mares.

The result of this earlier enquiry into the composition of British mares' milk attracted some attention from breeders, and more particularly from the breeders of thoroughbreds, as it appeared possible that some mares might have a natural tendency to secrete milk richer in mineral matter than others, and thus they might be expected to nourish their foals better than would mares yielding milk comparatively poor in ash. The mineral-rich milk might cause quicker growth and possibly also the formation of heavier osseous tissue. It will be remembered in this connexion that Bunge *et al.* (2) had shown that rapidity of early growth is directly proportional to the "strength" of the milk, i.e. with its content of protein and ash.

If therefore some mares secreted milk richer in mineral matter than others, a further investigation into the lactation of mares was obviously desirable. Accordingly milk from Thoroughbred mares was collected during the years 1931, 1932, 1933 and 1934, and the percentage of mineral matter was determined in 125 samples. Among the first of these were fifteen from one stud which included some of the best of the thoroughbred stock, and when the percentage of ash was determined in the samples wide differences were found to occur (Table I).

Table I. *The percentage of ash in the milk of fifteen thoroughbreds*

Mare	Days *post partum*	Ash %	Mare	Days *post partum*	Ash %
A	146	0·23	I	199	0·19
B	127	0·24	J	169	0·23
C	153	0·24	K	159	0·24
D	122	0·26	L	170	0·24
E	139	0·26	M	191	0·25
F	98	0·28	N	102	0·30
G	111	0·29	O	140	0·31
H	54	0·40		Mean	0·264

The average percentage of ash previously determined for the milk of fourteen thoroughbreds was 0·38, with a maximum of 0·53 per cent and a minimum of 0·28 per cent. Clearly therefore considerable variations in the percentage of mineral matter may be encountered in mares' milks. The primary purpose of this investigation was to determine the cause of these variations.

During the course of the enquiry several samples of milk were received from individual mares, the milk being drawn at intervals during the lactation periods, and when two or more samples were received from the same mare it was invariably found that later samples contained smaller quantities of ash. Since samples drawn after the first always contained a lower percentage of ash, it was possible that the ash actually decreased appreciably with the advance in lactation. Thus Diagram 1, which is representative, shows how the percentages

of ash, CaO and P_2O_5, found in the milk of four samples withdrawn from a thoroughbred mare on the 14th, 69th, 120th and 162nd days *post partum*, decreased with the advance in lactation.

A possible explanation of the differences in the percentage of ash in the milk of the mares (see Table I) might therefore be that while all the samples were obtained during the same month (July), the period of the lactation in which they were drawn might be markedly different. Thus, low values might be associated with the period *post partum* rather than with any peculiarity of

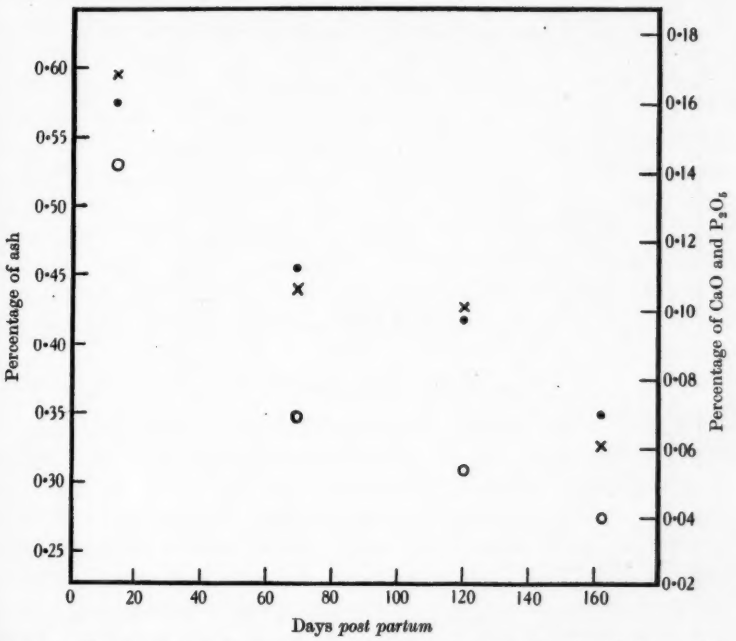

Diagram 1. The percentages of ash, CaO and P_2O_5 in four samples of milk drawn from a thoroughbred mare. The diagram illustrates how the percentages decrease as lactation advances.

Ash × CaO • P_2O_5 ○

the individual. That this was so is evident when the percentage of ash is considered in relation to the number of days *post partum*. Mare "H", with the shortest period *post partum*, had the highest percentage of ash in her milk, while mare "I", with the longest period, had the lowest percentage.

Moreover, 104 ash determinations made from the milk of thoroughbreds during the periods 1931, 1932, 1933 and 1934 were plotted against the days *post partum* (Diagram 2). The same definite tendency for the ash values to fall with advance in lactation is noted.

A reduction in the percentage of mineral matter with the advance of lactation has not been found to occur in the milk of any other domestic animal.

The percentage of ash in cow's milk undergoes little change in the post-colostral period, and what alteration there is is towards an increase rather than a decrease. It has been shown(3, 4) that with very prolonged uninterrupted lactations the percentage of ash in the milk is definitely higher than the average.

Information regarding variations in the percentage of the milk constituents in the case of ewes is scanty, but Godden & Puddy(5) have shown that there is a tendency for the percentage of the ash and the total nitrogen to increase with advance in lactation. In connexion with the present investigation, the per-

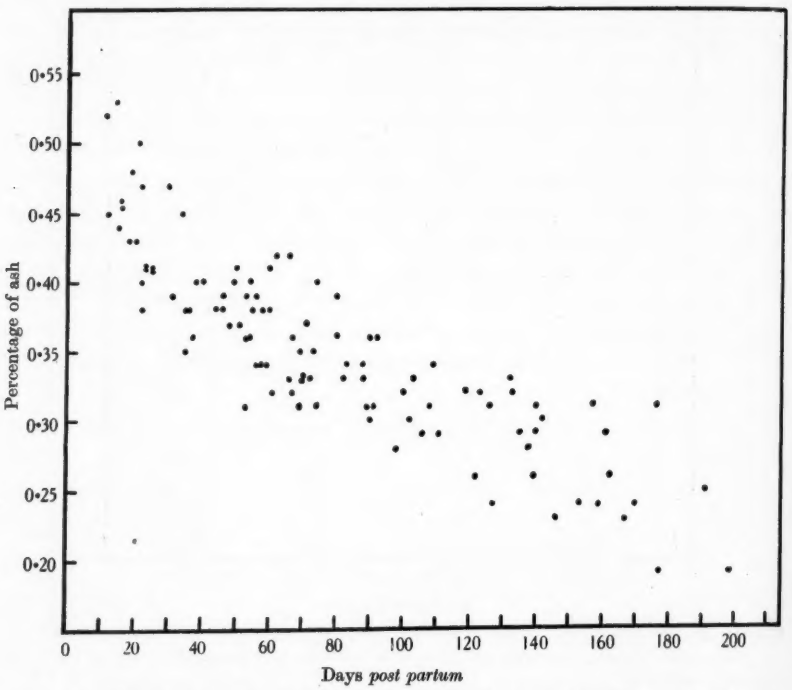

Diagram 2. The percentage of ash in 104 samples of milk drawn from Thoroughbred mares during, 1931, 1932, 1933 and 1934 shown in relation to the period of lactation when the samples were taken.

centage of ash in the milk of twenty-three ewes was determined at various intervals after lambing, but no definite variation was noted in the course of the lactations.

Hughes & Hart(6) found that the percentages of ash and protein in sow's milk increase during the course of lactation; this might be expected in view of the requirements of a rapidly growing litter in contrast to that of a slower growing single suckling.

While a great many analyses of human milk have been made by different

observers, there appears to be a singular lack of information regarding variations in the composition of the milk throughout the whole lactation period of individuals.

Evidence produced by the following authors—Wardlaw & Dart(7), Lowenfeld *et al.*(8), Marion Bell(9) and Widdows *et al.*(10)—suggests that in the case of nursing women the protein and ash of human milk also decrease very slightly as lactation advances.

As no systematic study of mare's milk had hitherto been carried out throughout a complete lactation, it was decided to make periodic analyses of the milk from the time of foaling to weaning, the mares being kept under natural conditions at grass. Periodic sampling of the milk of cows and sheep is a simple procedure, particularly with cows when the calves are completely separated from their dams, which are then hand-milked, but this is not the case with mares, especially since breeders usually resent interference with the suckling of the foals. Thus, to make the desired observations it was necessary to have complete charge of the mare. For this purpose Prof. Crew, Director, Animal Genetics Department, University of Edinburgh, kindly granted facilities for some Shetland ponies to be milked throughout the time they were nursing foals. Three ponies were used in 1932, three in 1933 and two in 1934. One Clydesdale mare was also available during the whole of her lactation.

METHOD OF SAMPLING

During the early part of lactation the ponies were milked daily, but after the first 3 weeks sampling was done weekly; the two ponies used in 1934 were milked at fortnightly intervals. To obtain the necessary quantity of milk the foals were separated from their mothers for 2 hours, always at the same period of the day. Both sides of the udder were then "stripped" as free from milk as possible and the milk from the two sides mixed. This method of obtaining samples was considered likely to give the most reliable results, because even were it possible to remove the foals altogether and to resort solely to hand milking, the udder of a mare would have to be emptied at least every 3 hours to avoid "pressure" milk. It is believed that the method adopted gave representative samples.

Analysis of the milk was begun within an hour of milking the mares.

ANALYTICAL TECHNIQUE

Total solids. 10 ml. samples were evaporated in flat silica basins on a water-bath and then kept in a thermostatically controlled electric oven at 100° C. until the weight became constant.

Protein. The protein was estimated from 5 ml. samples by the usual Kjeldahl method. The conversion factor used for the nitrogen was 6·39.

Lactose was determined "by difference".

Fat. The fat was estimated by the Gerber method, which was found to give satisfactory results.

Ash. The solids were incinerated in an electric furnace, at a temperature of approximately 600° C.

P_2O_5. Phosphoric anhydride was determined on 5 ml. milk by the Richards & Godden modification of the Pemberton-Newmann method (11).

Chlorine. The chlorine was determined on 10 ml. samples of milk by the method recommended by Davies(12).

Calcium. The calcium was determined volumetrically after precipitation as the oxalate.

The Hortvet apparatus was used to determine the *freezing-point*, and a Cambridge potentiometer with a glass electrode for the *p*H.

The percentage of ash in mare's milk

In 1932 the percentage of ash was determined in the milk of three Shetland ponies from parturition to weaning. They were kept under natural conditions at grass without receiving any artificial food. The foals of mares Nos. 1 and 2 were weaned on the 128th and 115th day *post partum* respectively, but the secretion of pony No. 3 unaccountably ceased on the 64th day after foaling. The results are given in Table II and show that a decrease in the percentage of ash occurred throughout the lactation period in the milk of each animal, the values at the close of lactation being approximately half those at the beginning of the post-colostral period.

As a similar fall in the percentage of mineral matter throughout lactation

Table II. *Percentages of ash in the milk of three Shetland ponies throughout complete lactation periods* (1932)

Days *post partum*	Ash %	Days *post partum*	Ash %	Days *post partum*	Ash %
6	0·549	2	0·539	4	0·608
9	0·521	5	0·600	7	0·570
13	0·487	9	0·538	17	0·474
16	0·462	12	0·515	21	0·443
20	0·473	16	0·475	24	0·421
23	0·430	19	0·450	28	0·379
27	0·392	23	0·421	31	0·378
30	0·394	26	0·413	35	0·326
34	0·394	30	0·394	39	0·364
37	0·351	33	0·383	43	0·330
41	0·353	37	0·379	46	0·308
44	0·357	41	0·403	50	0·307
48	0·331	45	0·372	53	0·300
51	0·388	48	0·346	57	0·273
55	0·344	52	0·367	60	0·283
58	0·331	55	0·360	64	0·305
62	0·324	59	0·311	Secretion ceased	
65	0·291	62	0·289		
69	0·283	66	0·356		
72	0·289	69	0·350		
79	0·283	73	0·321		
83	0·277	80	0·314		
86	0·278	83	0·328		
90	0·267	90	0·320		
93	0·293	94	0·299		
100	0·281	97	0·277		
104	0·285	101	0·294		
107	0·267	113	0·286		
111	0·266	114	0·268		
124	0·259	115	0·280		
125	0·280				
128	0·289				

had not hitherto been found with any domestic animal, the work was repeated during the following season using three different Shetland ponies. In addition to acting as a check on the previous findings, a repetition during a different season tended to eliminate the possibility that the quality and quantity of the grazing influenced the results. The opportunity was taken to determine the percentage of total solids, protein, fat, lactose, calcium, phosphorus and chlorine as well as the total ash (Tables III–V).

The systematic periodical analysis of the milk of the three ponies in 1933 confirmed the results obtained in 1932. The ash in the milk of the three mares (Nos. 4, 5 and 6) fell during the post-colostral period from 0·464 (5th day) to 0·235 per cent (168th day), with lower values intermediate towards the end of lactation; from 0·505 (5th day) to 0·243 per cent (157th day) and from 0·557 (18th day) to 0·210 per cent on the 143rd day *post partum*.

In 1934 milk was obtained from two other Shetland ponies, but these could not be milked until the 39th and 59th days after foaling; similar reductions in the percentage of ash were again found (Table VI).

The percentages of total ash of the 197 samples of milk drawn from the eight Shetland ponies in the present investigation are shown in relation to the period of lactation in Diagram 3.

As it was obviously important to ascertain whether this change occurred in the percentage of the ash of the milk of the heavy breeds, a Clydesdale mare was milked from the day of parturition to weaning (112th day); the milk was sampled daily for the first fortnight and then at 7-day intervals. While considerable fluctuations in the percentages of the constituents were noted, probably at least in part due to the difficulty sometimes experienced in obtaining milk, there being occasions when milk could only be withdrawn when the foal was sucking the opposite teat, it was found that a similar and quite definite decrease in the percentage of ash occurred, from 0·649 per cent on the 5th day after foaling to 0·324 per cent on the 112th day (Table VII).

The percentage of ash in the milk has therefore been found to decrease throughout lactation periods in eight Shetland ponies and one Clydesdale mare, and also in the case of several thoroughbred mares when samples of their milk were analysed at intervals while they were suckling foals.

It is therefore not possible to give an "average" value for the ash of mare's milk, as the percentage in the milk of any individual mare decreases throughout lactation. Thus an explanation has been found for the marked differences in the percentage of ash which have been submitted by breeders for analytical examination.

Reference has been made earlier in this communication to the variation in the percentage of ash that was found to be present in samples of milk obtained from thoroughbred mares. In view of the fact now determined that the percentage of mineral matter in the milk of a mare is not a constant value throughout lactation, an erroneous conception of the significance of values higher or lower than an "average" has been previously entertained.

Table III. *Percentage composition of the milk of Shetland pony No.* 4 (1933) *throughout complete lactation. Foaled* 4 *May* 1933

Days *post partum*	Total solids	Solids-not-fat	Protein	Fat	Lactose	Chlorine-lactose index	Ash	CaO in the milk %	CaO in the ash %	P_2O_5 in the milk %	P_2O_5 in the ash %	Cl in the milk %	Cl in the ash %	CaO/P_2O_5
1	12·20	10·50	3·11	1·70	6·92	0·732	0·531	0·145	27·3	0·201	37·9	0·050	9·4	1 : 1·39
2	10·72	9·52	2·58	1·20	6·45	0·620	0·492	0·145	29·5	0·197	40·0	0·040	8·1	1 : 1·36
4	11·58	9·98	2·64	1·60	6·86	0·598	0·475	0·153	32·2	0·166	34·9	0·041	8·6	1 : 1·08
5	11·05	9·65	2·49	1·40	6·70	0·492	0·464	0·149	38·1	0·164	35·3	0·033	7·1	1 : 1·10
6	10·42	9·77	2·46	0·65	6·84	0·511	0·467	0·149	31·9	0·162	34·7	0·035	7·5	1 : 1·09
7	10·53	9·68	2·33	0·85	6·91	0·347	0·442	0·142	32·1	0·143	32·4	0·033	7·5	1 : 1·01
8	10·40	9·75	2·39	0·65	6·88	0·377	0·476	0·153	32·2	0·145	30·5	0·026	5·5	1 : 0·95
9	10·40	9·80	2·56	0·60	6·79	0·412	0·450	0·153	34·1	0·142	31·2	0·028	6·2	1 : 0·93
11	10·35	9·65	2·21	0·70	7·00	0·342	0·440	0·139	31·7	0·142	32·2	0·024	5·5	1 : 1·02
12	10·24	9·39	2·11	0·85	6·89	0·362	0·438	0·134	30·9	0·128	29·3	0·025	5·6	1 : 0·96
13	9·85	9·40	1·87	0·45	7·15	0·363	0·384	0·125	32·5	—	—	0·026	6·6	—
14	9·66	9·11	1·81	0·55	6·94	0·405	0·355	0·115	32·3	—	—	0·028	7·8	—
15	10·11	9·26	1·61	0·85	7·30	0·370	0·351	0·110	31·4	0·109	31·1	0·027	7·7	1 : 0·99
16	10·16	9·31	2·01	0·85	6·92	0·433	0·378	0·121	32·0	0·111	29·3	0·030	7·8	1 : 0·92
19	10·75	9·70	2·16	1·05	7·16	0·391	0·369	0·138	37·6	—	—	0·028	7·5	—
21	11·40	9·40	2·07	2·00	7·03	0·355	0·296	0·132	44·5	0·103	34·9	0·025	8·4	1 : 0·78
28	11·17	9·67	—	1·50	—	—	0·325	0·120	37·0	0·108	33·2	0·023	7·2	1 : 0·89
36	11·04	9·76	2·42	1·28	6·98	0·315	0·361	0·140	38·8	0·127	35·8	0·022	6·1	1 : 0·91
43	10·12	9·52	1·90	0·60	7·36	0·244	0·253	0·113	44·8	0·100	39·5	0·018	7·0	1 : 0·89
50	12·34	10·04	2·38	2·30	7·35	0·244	0·313	0·121	38·7	0·137	43·9	0·018	5·8	1 : 1·13
57	10·57	9·57	2·33	1·00	6·94	0·288	0·301	0·118	39·1	0·121	40·1	0·020	6·5	1 : 1·03
64	10·44	9·09	2·06	1·35	6·75	0·266	0·283	0·109	38·6	0·104	36·8	0·018	6·3	1 : 0·95
71	10·09	9·49	1·97	0·60	7·36	0·244	0·264	0·099	37·7	0·092	34·8	0·018	6·8	1 : 0·93
78	10·06	9·26	1·78	0·80	7·23	0·250	0·250	0·095	38·0	0·088	35·2	0·018	7·2	1 : 0·93
85	10·58	9·58	—	1·00	—	—	0·254	0·099	39·0	0·096	37·8	0·018	7·1	1 : 0·97
92	11·32	9·02	1·78	2·30	6·97	0·230	0·274	0·087	31·8	0·091	33·2	0·016	5·8	1 : 1·05
99	10·14	9·36	—	0·78	—	—	0·254	0·087	34·3	0·082	32·3	0·011	4·3	1 : 0·94
106	10·61	9·49	2·02	1·12	7·22	0·193	0·247	0·091	36·9	0·096	38·9	0·014	5·7	1 : 1·06
113	10·29	9·44	1·84	0·85	7·37	0·162	0·234	0·086	36·8	0·086	36·8	0·012	5·1	1 : 1·00
120	10·45	9·45	1·96	1·00	7·25	0·234	0·235	0·088	37·5	0·088	37·5	0·017	7·2	1 : 1·00
127	10·78	9·38	1·78	1·40	7·38	0·162	0·215	0·070	32·6	0·068	31·6	0·012	5·6	1 : 0·97
133	10·10	8·90	1·87	1·20	6·81	0·280	0·220	0·077	35·0	0·075	34·1	0·019	8·6	1 : 0·97
140	11·26	9·56	2·08	1·70	7·25	0·275	0·225	0·082	36·5	0·088	39·1	0·020	8·9	1 : 1·07
147	10·91	9·51	1·99	1·40	7·30	—	0·220	0·079	35·9	0·072	32·7	—	—	1 : 0·91
154	11·60	9·65	2·06	1·95	7·36	0·272	0·230	0·080	34·8	0·073	31·7	0·020	8·7	1 : 0·91
161	10·36	9·26	1·78	1·10	7·29	—	—	—	—	—	—	—	—	—
168	11·93	9·43	2·04	2·50	7·15	0·307	0·235	0·080	34·1	0·071	30·2	0·022	9·4	1 : 0·89

Table IV. *Percentage composition of the milk of Shetland pony No.* 5 (1933) *throughout complete lactation. Foaled* 15 *May* 1933

Days *post partum*	Total solids	Solids-not-fat	Protein	Fat	Lactose	Chlorine-lactose index	Ash	CaO in the milk %	CaO in the ash %	P_2O_5 in the milk %	P_2O_5 in the ash %	Cl in the milk %	Cl in the ash %	CaO/P_2O_5
2	10·57	9·97	2·98	0·60	6·83	0·439	0·557	0·139	25·0	0·192	34·5	0·030	5·4	1 : 1·38
3	10·28	9·92	2·93	0·36	6·44	0·496	0·553	0·160	28·9	0·158	28·6	0·032	5·8	1 : 0·99
4	10·93	10·38	2·77	0·55	7·02	0·455	0·592	0·153	25·8	0·182	30·7	0·032	5·4	1 : 1·19
5	10·44	9·64	2·70	0·80	6·43	0·435	0·505	0·145	28·6	0·164	32·6	0·028	5·5	1 : 1·13
10	10·14	9·59	2·52	0·55	6·57	0·395	0·498	0·165	33·1	0·150	30·1	0·026	5·2	1 : 0·91
11	10·27	9·81	2·33	0·46	7·04	0·355	0·442	0·170	38·5	0·149	33·7	0·025	5·7	1 : 0·88
12	10·84	10·39	2·19	0·45	6·77	0·400	0·432	0·158	36·6	0·129	29·9	0·027	6·3	1 : 0·82
14	9·69	9·44	1·81	0·25	7·17	0·306	0·462	0·155	33·6	0·134	29·0	0·022	4·8	1 : 0·87
15	9·77	9·02	2·08	0·75	6·53	0·474	0·410	0·137	33·4	0·124	30·3	0·031	7·6	1 : 0·91
16	10·30	9·25	—	1·05	—	—	0·428	0·152	35·5	0·129	30·2	0·028	6·6	1 : 0·85
17	10·17	9·37	—	0·80	—	—	0·430	0·155	36·1	0·126	29·3	0·025	5·8	1 : 0·81
18	10·05	9·35	—	0·70	—	—	0·419	0·146	34·8	0·122	29·5	0·027	6·4	1 : 0·84
19	10·64	9·64	—	1·00	—	—	0·410	0·139	33·9	0·141	34·4	0·025	6·1	1 : 1·02
21	10·44	9·49	2·05	0·95	7·03	0·398	0·410	0·140	34·2	0·132	32·2	0·028	6·8	1 : 0·94
22	9·91	9·31	2·07	0·60	6·83	0·380	0·405	0·138	34·1	0·121	29·9	0·026	6·4	1 : 0·88
23	11·09	—	2·21	—	—	—	0·342	0·141	41·2	0·109	31·9	—	—	1 : 0·77
24	10·45	9·45	2·08	1·00	7·02	0·328	0·353	0·136	38·6	0·116	32·9	0·023	6·5	1 : 0·85
25	10·01	9·76	2·11	0·25	7·31	0·287	0·340	0·135	39·7	0·105	30·9	0·021	6·2	1 : 0·78
28	10·00	9·85	2·07	0·15	7·44	0·282	0·341	0·131	38·4	0·123	36·1	0·021	6·2	1 : 0·94
30	9·86	9·46	2·06	0·40	7·02	0·300	0·383	0·134	35·0	0·110	28·7	0·021	5·5	1 : 0·82
31	9·73	9·58	1·78	0·15	7·45	0·255	0·354	0·137	38·7	0·106	30·0	0·019	5·4	1 : 0·77
32	9·39	8·89	1·87	0·50	6·67	0·314	0·353	0·123	34·9	0·107	30·3	0·021	6·0	1 : 0·87
35	9·50	9·40	1·95	0·10	7·09	0·240	0·361	0·118	32·7	0·109	30·2	0·017	4·7	1 : 0·92
36	10·71	10·21	1·83	0·50	8·09	0·223	0·287	0·113	39·4	0·101	25·2	0·018	6·3	1 : 0·90
37	9·43	9·13	1·85	0·30	6·94	0·260	0·344	—	—	0·105	30·5	0·018	5·2	—
38	9·16	8·96	1·73	0·20	6·89	0·290	0·337	0·118	35·0	0·103	30·6	0·020	5·9	1 : 0·87
39	—	—	1·75	0·30	—	—	0·321	0·112	34·9	0·100	31·1	0·020	6·2	1 : 0·89
42	9·63	9·33	1·76	0·30	7·21	0·263	0·363	0·125	34·4	0·113	31·1	0·019	5·2	1 : 0·90
46	9·81	9·31	2·24	0·50	6·70	0·314	0·370	0·128	34·6	0·124	33·5	0·021	5·7	1 : 0·97
53	9·72	8·72	2·29	1·00	6·05	0·264	0·381	0·131	34·4	0·111	29·2	0·016	4·2	1 : 0·85
60	9·64	9·14	1·95	0·50	6·89	0·190	0·303	0·114	37·6	0·082	27·1	0·013	4·3	1 : 0·72
67	9·62	9·12	—	0·50	—	—	0·302	0·109	36·1	0·097	32·1	0·018	6·0	1 : 0·89
74	10·00	9·40	1·90	0·60	7·21	0·250	0·288	0·103	35·8	0·092	32·0	0·018	6·3	1 : 0·89
81	9·74	9·14	1·70	0·60	7·16	0·237	0·283	0·089	31·4	0·088	31·1	0·017	6·0	1 : 0·99
88	9·84	9·44	1·94	0·40	7·20	0·180	0·297	0·091	30·6	0·083	27·9	0·013	4·4	1 : 0·91
95	10·12	9·32	1·91	0·80	7·16	0·182	0·269	0·098	36·5	0·097	36·1	0·013	4·8	1 : 0·99
102	10·14	9·39	1·96	0·75	7·15	0·168	0·283	0·097	34·3	0·077	27·2	0·012	4·2	1 : 0·79
109	9·93	9·08	1·81	0·85	7·03	0·242	0·235	0·086	36·6	0·081	34·5	0·017	7·2	1 : 0·94
116	10·57	9·42	1·95	1·15	7·21	0·166	0·264	0·087	33·0	0·076	28·8	0·012	4·6	1 : 0·87
122	9·92	9·17	1·70	0·75	7·22	0·221	0·254	0·083	32·7	0·076	29·9	0·016	6·3	1 : 0·92
129	10·39	9·21	1·82	1·18	7·15	0·265	0·244	0·087	35·7	0·081	33·2	0·019	7·8	1 : 0·93
136	10·22	9·12	1·69	1·10	7·21	—	0·220	0·071	32·3	0·069	31·4	—	—	1 : 0·97
143	11·02	9·12	1·82	1·90	7·05	0·255	0·245	0·076	31·0	0·071	29·0	0·018	7·4	1 : 0·93
150	10·49	9·19	1·96	1·30	7·00	0·272	0·230	0·081	35·2	0·076	33·1	0·019	8·3	1 : 0·94
157	10·81	8·91	1·85	1·90	6·82	0·293	0·243	0·083	34·2	0·092	37·9	0·020	8·2	1 : 1·11

Table V. *Percentage composition of the milk of Shetland pony No.* 6 (1933) *throughout complete lactation. Foaled* 22 *May* 1933.

Days *post partum*	Total solids	Solids-not-fat	Protein	Fat	Lactose	Chlorine-lactose index	Ash	CaO in the milk %	CaO in the ash %	P_2O_5 in the milk %	P_2O_5 in the ash %	Cl in the milk %	Cl in the ash %	CaO/P_2O_5
1	15·41	10·26	3·51	5·15	6·30	1·062	0·450	0·119	26·5	—	—	0·067	14·9	—
2	13·58	9·43	3·45	4·15	5·44	—	0·537	0·127	23·7	—	—	—	—	—
18	11·86	10·26	3·15	1·60	6·55	0·427	0·557	0·205	36·8	0·211	37·9	0·028	5·0	1 : 1·03
25	10·56	10·36	2·72	0·20	7·16	0·307	0·478	0·202	42·3	0·180	37·7	0·022	4·6	1 : 0·89
32	11·73	9·63	2·49	2·10	6·71	—	0·429	0·171	39·9	0·144	33·6	—	—	1 : 0·84
39	10·12	9·32	1·98	0·80	7·02	—	0·316	0·121	38·3	0·096	30·4	—	—	1 : 0·79
46	10·03	8·98	2·04	1·05	6·61	0·272	0·329	0·118	35·9	0·107	32·6	0·018	5·5	1 : 0·91
53	9·77	9·27	1·99	0·50	6·95	0·202	0·327	0·119	36·4	0·112	34·3	0·014	4·3	1 : 0·94
60	9·49	9·09	1·66	0·40	7·17	0·223	0·259	0·096	37·1	0·083	32·0	0·016	6·2	1 : 0·86
70	10·26	9·36	1·82	0·90	7·26	0·207	0·284	0·102	35·9	0·091	32·0	0·015	5·3	1 : 0·89
74	9·96	9·26	1·94	0·70	7·02	0·185	0·299	0·100	33·5	0·101	33·8	0·013	4·3	1 : 1·01
81	9·72	9·42	1·97	0·30	7·17	0·139	0·283	0·096	33·9	0·099	35·0	0·010	3·5	1 : 1·03
88	10·35	9·17	1·81	1·18	7·11	0·155	0·253	0·096	38·0	0·084	33·2	0·011	4·4	1 : 0·88
95	10·23	9·38	1·82	0·85	7·32	0·150	0·244	0·085	34·9	0·081	33·2	0·011	4·5	1 : 0·95
102	10·71	9·36	1·92	1·35	7·19	0·167	0·250	0·089	35·6	0·095	38·0	0·012	4·8	1 : 1·07
109	10·54	9·54	1·86	1·00	7·46	0·134	0·224	0·088	39·3	0·084	37·5	0·010	4·5	1 : 0·96
115	10·57	9·57	1·69	1·00	7·63	0·190	0·246	0·083	33·7	0·075	30·5	0·014	5·7	1 : 0·90
122	10·08	9·13	1·78	0·95	7·14	0·238	0·210	0·071	33·8	0·073	34·8	0·017	8·1	1 : 1·03
129	10·81	9·41	1·97	1·40	7·22	—	0·220	0·073	33·2	0·080	36·4	—	—	1 : 1·10
136	10·47	9·12	1·93	1·35	6·94	0·216	0·245	0·076	31·0	0·078	31·8	0·015	6·1	1 : 1·03
143	10·28	9·38	1·95	0·90	7·22	0·222	0·210	0·081	38·6	0·079	37·6	0·016	7·6	1 : 0·98

Table VI. *Percentage composition of the milk of Shetland ponies Nos.* 7 *and* 8 (1934)

Days *post partum*	Total solids	Solids-not-fat	Protein	Fat	Lactose	Freezing-point	*p*H	Chlorine-lactose index	Ash	CaO in the milk %	CaO in the ash %	P_2O_5 in the milk %	P_2O_5 in the ash %	Cl in the milk %	Cl in the ash %	CaO/P_2O_5
Pony No. 7 (foaled 24 May 1934)																
39	9·63	8·70	2·27	0·93	6·09	−0·586°	7·00	0·312	0·334	0·113	33·8	0·103	30·8	0·019	5·7	1 : 0·91
68	10·41	9·91	2·56	0·50	7·03	—	—	—	0·321	0·113	35·2	0·095	29·6	—	—	1 : 0·84
81	10·26	9·86	2·12	0·40	7·46	−0·587°	7·00	0·268	0·282	0·099	35·1	0·085	30·2	0·020	7·1	1 : 0·86
95	11·14	9·54	2·17	1·60	7·12	—	—	0·295	0·272	0·100	36·8	0·081	29·8	0·021	7·7	1 : 0·81
109	9·81	9·21	1·78	0·60	7·17	—	6·95	0·237	0·262	0·092	35·1	0·081	30·9	0·017	6·5	1 : 0·88
123	—	—	2·02	0·90	—	—	—	—	0·267	0·090	33·7	0·078	29·2	0·017	6·4	1 : 0·87
137	10·25	9·75	2·01	0·50	7·45	−0·591°	7·05	0·256	0·293	0·092	31·4	0·082	28·0	0·019	6·5	1 : 0·89
152	10·57	9·77	2·16	0·80	7·32	—	7·14	0·287	0·291	0·087	29·9	0·066	22·7	0·021	7·2	1 : 0·76
Pony No. 8 (foaled 4 May 1934)																
59	11·32	9·82	2·70	1·50	6·69	−0·580°	6·70	0·344	0·407	0·133	32·7	0·128	31·4	0·023	5·7	1 : 0·96
73	10·88	10·46	2·56	0·42	7·52	—	6·84	—	0·378	—	—	0·116	30·7	—	—	—
88	10·67	10·12	2·77	0·55	6·96	—	—	—	0·388	0·125	32·2	0·116	29·9	—	—	1 : 0·93
101	9·89	9·49	2·09	0·40	7·10	—	6·90	0·268	0·302	0·113	37·4	0·092	30·5	0·019	6·3	1 : 0·81
115	10·95	9·72	2·25	1·23	6·87	—	—	0·262	0·296	0·117	39·5	0·099	33·5	0·018	6·1	1 : 0·85
129	9·40	9·10	1·82	0·30	7·01	—	7·05	0·242	0·272	0·091	33·5	0·093	34·2	0·017	6·3	1 : 1·02
143	—	—	1·97	0·60	—	—	—	—	0·306	0·095	31·1	0·095	31·1	0·016	5·2	1 : 1·00
157	11·03	10·33	1·86	0·70	8·19	−0·591°	7·10	0·232	0·276	0·094	34·1	0·090	32·6	0·019	6·9	1 : 0·96
172	10·18	9·57	2·10	0·61	7·16	−0·559°	7·10	0·363	0·315	0·096	30·5	0·090	28·6	0·026	8·3	1 : 0·94

This conclusion is confirmed by the work of Prowosnik(13), who examined 754 samples of mare's milk. In some cases milk from the same mare was analysed at intervals during lactation, but apparently Prowosnik did not notice the definite reduction in the percentage of ash. As, however, he recorded the date of parturition and the date of sampling, it has been possible to construct the table (Table VIII) to show that the percentage of ash decreased with the advance of lactation.

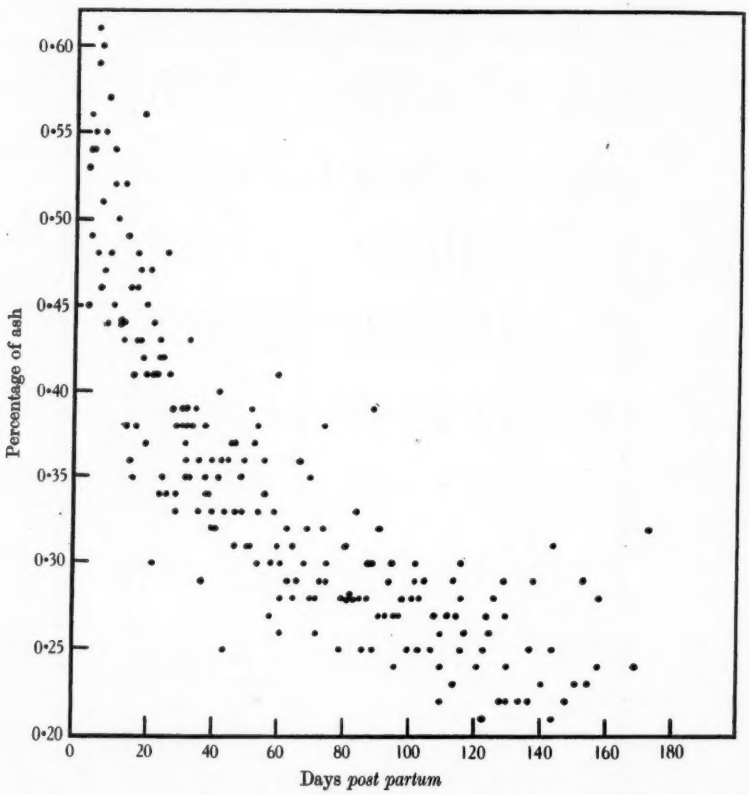

Diagram 3. The percentage of ash in 197 samples of milk drawn from eight Shetland ponies throughout their lactations shown in relation to the period of lactation when the samples were taken.

Statistical analysis

The data in Tables II–VI have been treated statistically by Mr J. E. Ritchie of Aberdeen. For the statistical analysis the total period of about 170 days was divided into 10-day subranges, giving seventeen subranges in all, and all observations of a constituent within each subrange were considered as a single group. As the first subrange, 1–10 days *post partum*, includes the colo-

Table VII. *Percentage composition of the milk of a Clydesdale mare* (1934) *throughout lactation. Foaled* 30 *May* 1934

Days *post partum*	Total solids	Solids-not-fat	Protein	Fat	Lactose	Freezing-point	*p*H	Chlorine-lactose index	Ash	CaO in the milk %	CaO in the ash %	P_2O_5 in the milk %	P_2O_5 in the ash %	Cl in the milk %	Cl in the ash %	CaO/P_2O_5
—	16·03	15·83	10·30	0·20	4·88	—	—	1·965	0·651	0·117	18·0	0·161	24·7	0·096	14·8	1 : 1·38
1	12·46	10·48	3·88	1·98	5·94	—	—	0·758	0·660	0·151	22·9	0·252	38·2	0·045	6·8	1 : 1·67
2	12·09	10·49	3·67	1·60	6·20	—	—	0·532	0·616	0·153	24·8	0·237	38·5	0·033	5·4	1 : 1·55
3	—	—	3·49	2·50	—	—	—	—	0·568	0·166	29·2	0·209	36·8	0·035	6·2	1 : 1·26
5	11·96	10·71	3·67	1·25	6·40	—	—	0·578	0·649	0·209	32·2	0·225	34·7	0·037	5·7	1 : 1·08
6	12·58	10·33	3·56	2·25	6·11	—	—	0·573	0·661	0·206	31·1	0·226	34·2	0·035	5·3	1 : 1·10
7	12·03	10·13	3·51	1·90	6·02	−0·550°	6·86	0·599	0·600	0·203	33·9	0·211	35·2	0·036	6·0	1 : 1·04
8	12·50	10·20	3·29	2·30	6·31	−0·555°	6·86	0·523	0·600	0·178	29·7	0·203	33·8	0·033	5·7	1 : 1·14
9	12·80	10·05	3·30	2·75	6·17	−0·555°	6·72	0·567	0·583	0·187	32·1	0·199	34·1	0·035	6·0	1 : 1·06
10	12·65	10·10	3·34	2·55	6·16	−0·556°	6·87	0·503	0·603	0·200	33·2	0·206	34·2	0·031	5·1	1 : 1·03
12	12·29	10·29	3·45	2·00	6·20	−0·556°	—	0·500	0·636	0·212	33·3	0·207	32·6	0·031	4·9	1 : 0·98
13	11·73	9·98	2·92	1·75	6·48	—	—	0·804	0·583	0·183	31·4	0·173	29·7	0·052	8·9	1 : 0·95
20	11·54	9·59	3·02	1·95	6·06	−0·566°	7·02	0·512	0·508	0·171	33·7	0·157	30·9	0·031	6·1	1 : 0·90
27	11·43	9·36	—	2·07	—	−0·563°	7·10	—	0·466	0·144	30·9	0·156	33·5	0·033	7·1	1 : 1·08
34	11·76	8·81	2·36	2·95	6·06	−0·558°	6·82	0·512	0·390	0·131	33·6	0·114	29·2	0·031	8·0	1 : 0·87
41	10·32	10·22	2·84	0·10	6·92	−0·568°	6·95	0·635	0·460	0·161	35·0	0·143	31·1	0·044	9·6	1 : 0·89
48	11·24	9·14	—	2·10	—	−0·572°	7·00	—	0·389	0·135	34·7	0·125	32·1	0·022	5·7	1 : 0·93
56	12·36	9·16	2·19	3·20	6·61	—	—	—	0·360	0·122	33·9	0·110	30·6	—	—	1 : 0·90
70	11·41	8·31	2·64	3·10	5·33	—	—	—	0·336	0·114	33·9	0·108	32·2	—	—	1 : 0·95
77	11·19	9·29	2·08	1·90	6·85	−0·564°	6·75	0·350	0·358	0·124	34·7	0·116	32·4	0·024	6·7	1 : 0·94
84	13·21	9·43	2·00	3·78	7·15	−0·561°	6·80	0·294	0·278	0·089	32·1	0·088	31·7	0·021	7·6	1 : 0·99
91	12·82	9·09	2·17	3·73	6·56	—	6·90	0·411	0·360	0·128	35·6	0·117	32·5	0·027	7·5	1 : 0·91
98	9·90	9·70	2·06	0·20	7·32	—	6·97	0·355	0·320	0·109	34·1	0·106	33·1	0·026	8·1	1 : 0·97
105	11·26	9·28	2·00	1·98	6·92	—	—	0·390	0·364	0·115	31·6	0·114	31·3	0·027	7·4	1 : 0·99
112	—	—	2·04	2·60	—	—	—	—	0·324	0·107	33·0	0·101	31·2	0·018	5·6	1 : 0·94

strum period the observations included in this group were omitted in the calculation of the regression equations. The mean values for successive time periods were calculated for each of the constituents and appropriate regression curves fitted to these means. The algebraic equations for these curves are tabulated in Table IX, and in Table X are shown the values for the observed means of the various constituents of the milk and those calculated from the equations of Table IX. These results are shown in Diagrams 4 and 5.

Table VIII. *Percentage of ash at various periods of lactation—constructed from Prowosnik's records*

Mare	Foaled	Date sampled	Days *post partum*	Ash %
No. 3	1. iv. 29	16. iv. 29	15	0·42
		17. v. 29	45	0·35
		18. vi. 29	78	0·28
		16. vii. 29	105	0·25
No. 5	13. iv. 29	24. iv. 29	11	—
		28. v. 29	45	0·42
		22. vi. 29	70	0·45
		22. vii. 29	100	0·42
		20. viii. 29	129	0·32
		17. ix. 29	157	0·30
		18. x. 29	188	0·27
No. 7	2. vi. 28	19. vii. 28	47	0·35
		11. viii. 28	70	0·32
		15. ix. 28	105	0·28
		20. x. 28	140	0·15
No. 9	16. v. 28	21. v. 28	5	0·64
		20. vi. 28	35	0·40
		18. vii. 28	63	0·34
		16. viii. 28	92	0·34
No. 10	6. vi. 29	18. vii. 29	42	0·39
		16. viii. 29	71	0·29
		12. ix. 29	100	0·22

Variation in other milk constituents during lactation

Total solids, solids-not-fat, lactose and protein

It will be observed from Tables IX and X and Diagrams 4 and 5 in case of the total solids, solids-not-fat, lactose and protein that the deviations from linearity were not statistically significant. The percentage of total solids gradually increased as the course of lactation proceeded, the total increase amounting to about 0·4 per cent, while the percentage of solids-not-fat fell slightly from about 9·5 to 9·35 per cent. The proportion of lactose increased from rather less than 7 per cent to about 7·3 per cent. The amount of protein in the milk diminished as the lactation proceeded and reached a minimum value of approximately 1·5 per cent.

Fat

The percentage of fat in the mare's milk increased considerably in a linear manner during the lactation period and, as will be seen from Table X, the average ranged from 0·7 to 1·2 per cent. The extreme ranges of the five ponies and the one Clydesdale mare were as follows:

Pony 4	0·45 – 2·50 per cent
„ 5	0·10 – 1·90 „ „
„ 6	0·20 – 5·15 „ „
„ 7	0·40 – 1·60 „ „
„ 8	0·30 – 1·50 „ „
Clydesdale	0·10 – 3·78 „ „

The very high value, 5·15 per cent, with pony 6 was for milk drawn on the first day *post partum*. It is probable that much of the variation found in the percentage of fat of different mares, and even during the lactation of an in-

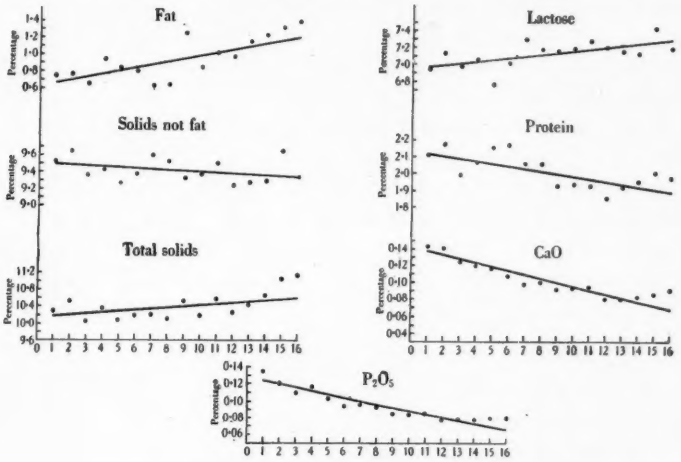

Diagram 4. Illustrating Table 10.

Table IX. *Regression equations giving the average values to be expected for various constituents, y, as the lactation proceeds*

$x = X/10$, where X = days *post partum*.

Constituent (y)	Equation
Fat	$y = 0{\cdot}611263 + 0{\cdot}036442\,x$
Solids-not-fat	$y = 9{\cdot}512470 - 0{\cdot}009807\,x$
Total solids	$y = 10{\cdot}149440 + 0{\cdot}027593\,x$
Ash	$y = 0{\cdot}467607 - 0{\cdot}031801\,x + 0{\cdot}001172\,x^2$
Lactose	$y = 6{\cdot}939291 + 0{\cdot}021411\,x$
Protein	$y = 2{\cdot}139141 - 0{\cdot}014975\,x$
Chlorine	$y = 0{\cdot}029878 - 0{\cdot}003011\,x + 0{\cdot}000152\,x^2$
CaO	$y = 0{\cdot}145632 - 0{\cdot}004669\,x$
P_2O_5	$y = 0{\cdot}130384 - 0{\cdot}003867\,x$
Chlorine-lactose index	$y = 0{\cdot}441550 - 0{\cdot}048575\,x + 0{\cdot}002466\,x^2$

dividual, was due to the method of sampling by which milk samples were drawn from udders containing varying quantities of milk. It has been found (Linton (1)) that foals do not thrive if the mare's milk contains an abnormally high percentage of fat, and in the course of this later work this observation has been confirmed by the analysis of similar milk samples sent for examination by veterinarians.

Calcium and phosphorus

It will be seen from Tables IX and X that within the limits of random sampling the regression of both calcium and phosphorus was linear in character, both decreasing similarly as lactation proceeded. During the colostral period, however, there is more phosphorus than calcium in the milk and in the ash. From the values shown in Table V it will be observed that pony 6 had a much higher percentage of calcium and phosphorus in its milk during the first month *post partum* than either pony 4 or 5. With ponies 4 and 5 the calcium ranged from 0·170 to 0·07 per cent and the phosphorus from 0·201 to 0·068 per cent. The milk of the Clydesdale mare contained slightly more—calcium (0·212–0·089 per cent), and phosphorus (0·252–0·088 per cent).

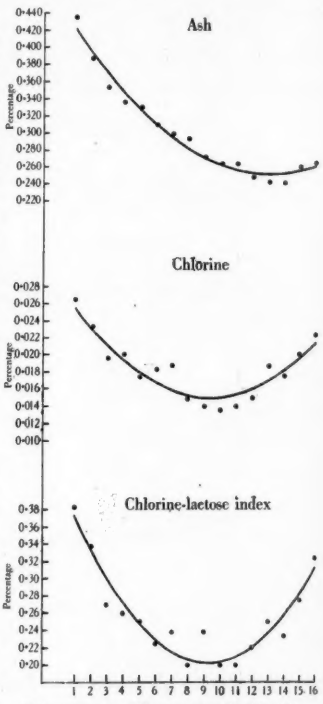

Diagram 5. Illustrating Table 10.

Chlorine

The amount of chlorine present in milk has assumed considerable importance since it has been shown that an appreciable increase above the normal indicates disturbance in milk secretion such as occurs in mastitis. In 1854 Rast (14) noted that mastitis milk had a salty taste and recommended tasting to detect this condition. An increase in the chlorine content in mastitis milk has been observed by many investigators; thus, Wiedmann (15, 16) stated that inflammation of the udder was accompanied by decrease in lactose, phosphorus and potassium with increases in sodium and chloride, the increase in sodium chloride being due to osmotic action when the lactose is decreased. Rosell (17) states that a chloride content of over 1·4 g./l. indicates the existence of mastitis. Tapernoux (18) considers the normal sodium chloride content of cow's milk to be 1·5–1·8 g./l. (0·09–0·11 g. Cl/100 ml.). Seelemann (19) states that in most cases of mastitis the chloride content was above 0·12 g./100 ml. The reliability of the so-called chloride test for mastitis is, however, questionable, especially since Blood & Rowlands (20) have shown that cow's milk may on occasion contain chloride in excess of the accepted normal range when, by bacteriological examination, there is no evidence of mastitis. In the case of the mare, however, the chlorine content of the milk shows very little individual fluctuation. The algebraic equation of the regression curve of the chlorine content of mare's milk is that of a parabola (Diagram 5). The regression curve for the total ash is

Table X. *Table showing observed mean values for constituents of mare's milk as compared with those calculated from the algebraic equations given in Table IX*

Period		Lactose percentage		Protein percentage		Chlorine percentage		CaO percentage		P_2O_5 percentage		Chlorine-lactose index		Fat percentage		Solids-not-fat percentage		Total solids percentage		Ash percentage	
No.	Range (days *post partum*)	Obs.	R.S.L.	Obs.	R.S.L.	Obs.	R.P.	Obs.	R.S.L.	Obs.	R.S.L.	Obs.	R.P.	Obs.	R.S.L.	Obs.	R.S.L.	Obs.	R.S.L.	Obs.	R.P.
1	11–20	6·947	6·972	2·113	2·116	0·0266	0·0256	0·1430	0·1384	0·1351	0·1244	0·3838	0·3722	0·749	0·668	9·517	9·497	10·292	10·192	0·4351	0·4211
2	21–30	7·130	6·994	2·172	2·101	0·0233	0·0232	0·1405	0·1337	0·1205	0·1205	0·3380	0·3337	0·772	0·704	9·649	9·488	10·535	10·220	0·3876	0·3941
3	31–40	6·985	7·015	1·987	2·086	0·0194	0·0211	0·1265	0·1291	0·1096	0·1167	0·2692	0·3002	0·650	0·741	9·365	9·478	10·035	10·247	0·3544	0·3695
4	41–50	7·055	7·037	2·065	2·071	0·0200	0·0193	0·1205	0·1244	0·1165	0·1128	0·2605	0·2716	0·945	0·777	9·425	9·468	10·375	10·275	0·3355	0·3472
5	51–60	6·772	7·058	2·155	2·056	0·0175	0·0178	0·1188	0·1197	0·1055	0·1089	0·2505	0·2479	0·822	0·814	9·272	9·458	10·088	10·303	0·3295	0·3272
6	61–70	7·022	7·079	2·172	2·041	0·0183	0·0167	0·1080	0·1151	0·0955	0·1051	0·2255	0·2292	0·805	0·850	9·380	9·448	10·205	10·330	0·3091	0·3096
7	71–80	7·295	7·101	2·045	2·026	0·0187	0·0158	0·0980	0·1104	0·0985	0·1012	0·2380	0·2154	0·625	0·886	9·605	9·438	10·215	10·358	0·2988	0·2943
8	81–90	7·172	7·122	2·055	2·011	0·0150	0·0152	0·1005	0·1057	0·0934	0·0973	0·2005	0·2065	0·634	0·923	9·534	9·429	10·141	10·385	0·2938	0·2814
9	91–100	7·155	7·144	1·930	1·996	0·0140	0·0149	0·0925	0·1010	0·0865	0·0935	0·2380	0·2026	1·265	0·959	9·325	9·419	10·535	10·413	0·2722	0·2708
10	101–110	7·184	7·165	1·934	1·981	0·0136	0·0150	0·0948	0·0964	0·0862	0·0896	0·2005	0·2036	0·848	0·996	9·376	9·409	10·198	10·441	0·2635	0·2625
11	111–120	7·275	7·187	1·925	1·966	0·0140	0·0153	0·0945	0·0917	0·0865	0·0857	0·2005	0·2095	1·025	1·032	9·505	9·399	10·575	10·468	0·2633	0·2566
12	121–130	7·205	7·208	1·848	1·951	0·0150	0·0160	0·0805	0·0870	0·0791	0·0819	0·2205	0·2203	0·976	1·069	9·238	9·389	10·255	10·496	0·2495	0·2531
13	131–140	7·155	7·229	1·925	1·936	0·0187	0·0169	0·0805	0·0824	0·0785	0·0780	0·2505	0·2361	1·165	1·105	9·285	9·380	10·455	10·523	0·2415	0·2519
14	141–150	7·130	7·251	1·945	1·921	0·0175	0·0182	0·0825	0·0777	0·0785	0·0741	0·2338	0·2568	1·225	1·142	9·305	9·370	10·655	10·551	0·2415	0·2530
15	151–160	7·430	7·272	2·005	1·906	0·0200	0·0197	0·0855	0·0730	0·0805	0·0702	0·2755	0·2825	1·330	1·178	9·655	9·360	11·055	10·579	0·2605	0·2565
16	161–	7·188	7·294	1·972	1·891	0·0225	0·0216	0·0905	0·0684	0·0805	0·0664	0·3255	0·3131	1·405	1·214	9·355	9·350	11·155	10·606	0·2655	0·2623

The above equations are those formulated by Mr J. E. Ritchie from the mean values submitted to him.

Obs. = observed. R.S.L. = regression straight line. R.P. = regression parabola.

also a parabola and not unlike that of the chlorine. With chlorine the percentage falls from about 0·25 at the beginning of the lactation period to about 0·15 near the middle and then rises until, at the end of the period under observation, it amounts to about 0·21 (see Diagram 5). The individual values show that if the percentage of chlorine is higher than 0·04 it may be assumed either that there is some abnormality in secretion or that the milk has not been properly sampled. Inefficient sampling may be due to reluctance on the part of the mare to "give down" her milk. This may in fact be the cause of the wider fluctuation noted in the case of the constituents of the Clydesdale's milk as compared with that of the ponies which were more tractable and which gave down their milk more readily. The tendency for the percentage of chlorine to increase towards the close of secretion has been observed in the cow. Allen (21) and Sharp & Struble (22) found that the chloride in the milk of healthy cows fell very rapidly during the first few days of the lactation period, reached a minimum, increased slightly during the first 60 per cent of the lactation period and then increased more rapidly toward the end of the period.

Chlorine-lactose index

While an abnormal chloride content in cow's milk is used by many investigators to differentiate between normal milk and mastitis milk, the ratio of chlorine to lactose is considered by some to give a more definite indication of abnormality. Porcher (23) demonstrated that an inverse ratio existed between the sodium chloride and lactose contents of milk.

Koestler (24) suggested the use of the chlorine-lactose figure or "index" (obtained by multiplying by 100 the ratio of percentage chlorine to percentage lactose) for the detection of abnormal milk; he found that the index increased with the intensity of the disturbance in the udder and obtained a range from 0·049 to a maximum of 15, which was for pathological milk. Koestler also stated that the ratio of chloride to phosphoric acid in the ash might serve to detect altered milk. Weiss (25) found the minimum value of the index to be 2, and the maximum to be 12·12 in a case of mastitis, and he considers that if a mixed milk has an index over 2·7 it is to be strongly suspected of containing a pathologically secreted milk. But he adds that for routine analysis the chlorine value alone may be used to indicate normal milk. Zaribnicky (26) considered the maximum value of the "index" for normal cow's milk to be 2·0–2·5. A range from 2 to 3·5 is considered to cover the normal value of the index in cow's milk.

For the correct determination of the chloride content and the chlorine-lactose index the method of sampling appears to be important because it has been shown that the chloride content is higher in the first-drawn milk than in the middle milk or the strippings in the case of cows affected with mastitis (Lorenzen (27), Seelemann (19)). Seelemann also determined the chloride content in the first-drawn milk and in the strippings from 585 healthy quarters and

obtained a higher percentage of chlorine in the first milk. It is clear, therefore, that if the chlorine or the chlorine-lactose index are used as guides in judging the abnormality of milk, the method of sampling must be controlled.

The chlorine-lactose index in mare's milk

As the primary purpose of this investigation was to attempt to define normal mare's milk, the chlorine-lactose index has been determined throughout lactation periods in the Shetland ponies, the Clydesdale mare and in numerous samples of milk submitted for examination. Since the chlorine regression is parabolic and that for lactose linear, it is to be expected that the regression for the chlorine-lactose index would also be parabolic, and this is clearly shown in Table X and Diagram 5. In fact the regression curve is very similar to that for chlorine. The normal index is much lower for mares than it is for cows. In the milk of five ponies the maximum normal range in the post-colostral period was found to be 0·51 to 0·13, and in the case of the Clydesdale mare, 0·60 to 0·29. Throughout the lactation periods the index was definitely higher in the milk of the Clydesdale than in any of the ponies. During the colostral period the index is much higher than in the later milk, and it may fall rapidly during the first 5 days *post partum*. As is shown in Table VII, an index as high as 1·97 was determined for the Clydesdale mare on the day of parturition. It may also be much higher than normal on the first day *post partum*, as in the case of pony 6, where the value was found to be 1·06.

If the colostral period is excluded, the normal chlorine-lactose index for mares may be considered to be between 0·12 and 0·50.

Abnormal chlorine-lactose indices

Abnormal indices have been noted in milk samples sent by veterinarians for investigation. For example, milk was drawn from a young Shire mare 1 month after foaling, the month-old foal suffering from diarrhoea. On analysis the milk was found to have the following composition:

Total solids	Solids-not-fat	Protein	Fat	Lactose	Ash	CaO	P_2O_5	Cl
9·65	6·65	2·44	3·00	3·61	0·601	0·100	0·057	0·201

The milk contained approximately three times the normal amount of fat; the lactose was very low; the ash higher than normal; the calcium rather low; the phosphorus approximately half the normal value, while the chlorine was very high. The chlorine-lactose index was 5·6 instead of approximately 0·5. The ratio of lime to phosphate was 1 : 0·57, whereas the normal ratio for the milk of a mare 1 month *post partum* is 1 : 1. The normal percentage of chlorine in the ash of the milk of a Clydesdale mare for a similar *post partum* period is 5–8 per cent and the normal percentage of P_2O_5 in the ash 30–34 per cent. In this case the values for the Cl and P_2O_5 are practically reversed, being 33·5 and 9·5 per cent respectively. This supports Koestler's statement that the ratio of chloride to phosphoric acid in the ash might serve to detect altered milk.

In this instance there was no evidence of bacterial infection of the udder, and it was believed that the abnormality of the milk, for which no cause could be found, was of a temporary nature. That this was the case is shown by the analysis of a sample obtained 3 days later which was found to be normal:

Total solids	Solids-not-fat	Protein	Fat	Lactose	Ash	CaO	P_2O_5	Cl
10·22	9·17	2·37	1·05	6·26	0·535	0·172	0·170	0·033

The chlorine-lactose index, 0·53, was normal, while the percentages of CaO, P_2O_5 and Cl in the ash, being 32·2, 31·8 and 6·2 respectively, indicated no departure from the normal. Thus confirmation is given to the findings of Blood & Rowlands(20) for cow's milk.

Abnormal chlorine-lactose indices were also encountered on two occasions during the weekly analysis of the milk of the Clydesdale mare. On the 13th day *post partum* the index was 0·804, and the percentage of Cl 0·052. There was no other divergence from the normal, but it was noted at the time of sampling that there was only a small quantity of milk in the udder. On the 41st day *post partum* the percentage of Cl was 0·044 and the index 0·625, and for some reason unknown the milk on this date differed considerably from both the earlier and later weekly samples. The solids-not-fat, protein, lactose, ash, P_2O_5, CaO and Cl were all higher than the average, while the percentage of fat was very low.

The occurrence of these temporary abnormalities clearly indicates that single samples of mare's milk may give misleading analytical results.

Percentages of the calcium, phosphorus and chlorine in the ash

Calcium and phosphorus

The percentages of calcium and phosphorus in the milk ash were not found to change to any appreciable extent during the secretion of milk in the post-colostral period, except that in the milk of the Clydesdale mare the percentage of phosphorus in the ash was higher in the earlier part of lactation than later. During the colostral period the percentage of calcium in the ash was much less than in normal milk.

Chlorine

The percentages of chlorine in the ash did not show the same fluctuation as occurred with the calcium and phosphorus. There appears to be a tendency for the percentage to increase towards the end of lactation. The values for the percentage of calcium, phosphorus and chlorine found in the ash throughout the lactation period are given in Tables III, IV, V, VI and VII.

Calcium-phosphorus ratio

The ratio of CaO to P_2O_5 in mare's milk has been found to be 1 : 1 by previous workers, whose opinions were based on occasional analyses. The present investigation confirms the previous observations. In the case of the

Clydesdale mare the average ratio from the 5th to the 112th day (day of weaning) is 1 : 0·99; variation is slight, and the extreme ranges noted are 1 : 0·87 to 1 : 1·14. The average ratio for each of the three ponies 4, 5 and 6 was found to be 1 : 0·96, 1 : 0·89 and 1 : 0·94 respectively.

In the case of pony 4 the ratio was greater than 1 : 0·9 on only four occasions, and of these three were 1 : 0·89 and one 1 : 0·78. In pony 5, however, there was greater variation, the range being from 1 : 0·72 to 1 : 1·13, but in the case of pony 6 the ratio was more constant.

It may be concluded therefore that the ratio of CaO to P_2O_5 in mare's milk throughout the post-colostral period is 1 : 0·85 to 1 : 1. As this holds throughout the whole post-colostral lactation period, it may be assumed that the optimum ratio CaO/P_2O_5 in the diet for growing horses is the same.

THE REACTION (*p*H) OF MARE'S MILK

The *p*H of the milk was determined within an hour after withdrawal at various periods during the lactation of the Clydesdale mare and the ponies 7 and 9. In the milk of the Clydesdale the range was 6·73 to 7·10, and in the milk of the ponies 6·70 to 7·14 during the post-colostral period of lactation.

FREEZING-POINT OF MILK

The freezing-point was determined at the same time as the *p*H. For the Clydesdale mare the range was from −0·550 to −0·572°, but in the case of the Shetland ponies, in the comparatively few observations it was possible to make, the depression of the freezing-point was found to be slightly greater, the range being −0·580 to −0·591° for five determinations, but on one occasion a value of −0·559° was recorded. No explanation is offered for the apparently greater depression of the freezing-point in the ponies' milk than in that of the Clydesdale mare's milk. These observations agree well with those of Winter (28), who gives the value of −0·57° for the milk of a mare.

THE LACTATION OF A CLYDESDALE MARE

For comparison with the milk of ponies the milk of a Clydesdale mare was analysed periodically from the day of parturition to weaning, the 112th day *post partum*. Milk was withdrawn from the udder daily during the first fortnight and thereafter at weekly or fortnightly intervals. The gradations in the milk constituents were found to agree closely with those of the milk of ponies.

In a previous paper (Linton (1)) it was stated that the milk of the heavy breeds of mares contains a higher percentage of ash than the milk of the lighter breeds. The present investigation confirms this statement, since it was found that the percentage of ash ranged from 0·66 to 0·32 in the milk of the Clydesdale mare from the 5th to the 112th day (day of weaning), whereas for a similar period the average figures for the ponies' milks are 0·54–0·26 per cent. The percentage of protein is also slightly higher, particularly in the early part of

lactation. The percentages of calcium, phosphorus and chlorine in the ash are the same as those in the ash of pony's milk, but the percentages in the milk are greater. The analytical results are recorded in Table VII.

Colostrum

The colostrum of three ponies and one Clydesdale mare was analysed, but it was only possible to obtain the milk secreted actually on the day of parturition from one mare, the Clydesdale. The results obtained confirm previous observations (Linton (1)). The mean values of the various constituents for the first 10-day period after foaling are:

Fat	1·387	Protein	2·761
Solids-not-fat	9·856	Chlorine	0·0358
Total solids	11·243	CaO	0·1465
Ash	0·5188	P_2O_5	0·1666
Lactose	6·619	Chlorine-lactose index	0·5265

The percentage of fat varied and an occasional high percentage was again found to occur (pony 6, Table V). While the percentage of lactose is lower than normal, the very low values previously noted have not been found in the colostrum of these four animals. The ash content was again high.

Calcium

Of the ash constituents determined, the calcium was found to be low at parturition and to increase steadily, reaching its maximum value at the end of the colostral period. Thus, the colostrum of the Clydesdale mare contained only 0·117 per cent CaO on the day of parturition, but it had increased to 0·209 per cent on the 5th day *post partum*. In the milk of two ponies the values rose from 0·145 to 0·152 per cent (1st to 4th day) and from 0·139 to 0·153 per cent (2nd to 4th day). An interesting feature of mare's colostrum is the low proportion of calcium in the ash as in the milk. Thus, while the average percentage of calcium (CaO) of the ash in the post-colostral period was 33–35 per cent, the percentage of ash in the first-drawn milk (day of parturition) of the Clydesdale was only 18·5, after which it steadily rose to reach 33 per cent on the 5th day *post partum*. The percentage of calcium in the colostrum and in the ash of the colostrum of the ponies was also found to be low. The percentage of calcium in the colostrum of cows is, of course, high.

Phosphorus

While the percentage of phosphorus in the milk and in the ash is higher during the colostral period than later, this is not so with the milk which was drawn from the Clydesdale mare on the day that she foaled. The percentage of the ash was then found to be at its lowest value, while the percentage in the milk, though lower than that of the succeeding day, was the same as that found at about 14 days *post partum*. With the exception of this single low value found on the day of parturition, the phosphorus in the milk is at a higher level during

the secretion of colostrum than during the secretion of normal milk. The low calcium and high phosphorus is reflected in the wider ratio CaO/P_2O_5, which is apparently characteristic of mare's colostrum. In the colostrum and in the ash of the colostrum of the cow the phosphorus is also high.

Chlorine

The percentage of chlorine in the milk is greater in colostrum than in the later secretion. The milk from the Clydesdale mare which was drawn on the day of parturition contained 0·096 per cent; on the 1st day *post partum* the Cl had decreased to 0·045 and on the 2nd day to 0·033 per cent, this level being maintained to the 43rd day. The colostral milk of two ponies was also found to be rich in chlorine on the 1st day *post partum*, 0·067 and 0·054 per cent. The percentage of chlorine in the ash may also be much higher than normal at the onset of lactation.

THE INFLUENCE OF INTRA-MAMMARY PRESSURE ON THE COMPOSITION OF MILK WITH SPECIAL REFERENCE TO THE MARE AND COW

While the natural weaning of suckled animals is a gradual process, the consumption of milk decreasing as the consumption of other food increases, the final and actual weaning of foals is effected by complete separation of the foal from the dam instead of allowing the foal to return to its mother at intervals. The method has been generally adopted because it has been found to cause less mental disturbance to the dam. Thus it follows that weaning is usually effected while milk secretion is still active and, therefore, milk may accumulate in the udder under pressure which will, of course, vary with the rate of secretion and the frequency with which the udder is emptied.

During this investigation several samples of milk which had been drawn when the foals were weaned were sent by breeders to ascertain whether it was of good quality. Analysis of these samples frequently revealed great abnormality in the composition, and it was presumed that the alteration in the character of the milk was caused by its accumulation under pressure, with resultant cessation of secretion and possibly resorption of some of the constituents.

Very few observations have been made regarding the chemical composition of "pressure" milk. Petersen & Rigor(29) found that as pressure developed in the gland of the cow the rate of secretion decreased until a pressure equal to 25 mm. Hg was developed, when secretion was checked and resorption set in. They then studied the changes which took place in the composition of the milk when it was kept in the udder for 24, 36 and 120 hours as well as for the normal 12-hour period. They found that there was an increase in the amount of milk produced for the 24- and 36-hour periods, then a marked decrease at the end of the 120-hour period, indicating that resorption of the milk had taken place in that time. Retention of milk in the udder had little influence upon the fat content for the 24- and 36-hour periods, but when the milk was retained for

120 hours there was a marked decrease in the percentage. The protein content increased as the period during which the milk was retained was extended. These authors pointed out, however, that the most striking effect of retention of milk in the udder is the decrease in the lactose content, from approximately 5 per cent to less than 1 per cent when the animal was left unmilked for 120 hours. The reduction in total solids was due chiefly to a decrease in lactose and fat. The ash increased, reaching a maximum in 30 hours. Both calcium and phosphorus decreased, the minimum values—slightly more than half the normal—being reached after 120 hours. A large increase in chlorine was noted. The increase in chlorine and decrease in lactose is taken as an indication that the milk is coming into equilibrium with the blood as far as the solutes are concerned.

The changes which occur in milk when it is retained in the udder under pressure are of interest not merely because their recognition would readily enable a distinction to be made between milk produced under normal and abnormal conditions, but also because it is hoped that a further study of the phenomena may establish a chemical standard for the detection of "overstocking" in cows which are left unmilked for lengthy periods prior to exposure for sale. It was for this purpose, as well as to make a comparison between "pressure" milk of cows and that of mares that, in 1933, the writer, then unaware of Petersen & Rigor's work, studied the changes which occurred in the milk of three cows when they were left unmilked for 24, 36 and 48 hours respectively. In each case the milk was analysed at a normal milking period previous to the experiment. Table XI gives the analytical data.

Table XI. *The effect of intra-mammary pressure on the percentage composition of cow's milk*

Cow	Period hours	Total solids	Solids-not-fat	Protein	Fat	Lactose	Ash	CaO	P_2O_5	Cl	Chlorine lactose index
1	12	14·07	8·77	3·32	5·30	4·69	0·760	0·195	0·246	0·095	2·02
	24	13·97	7·92	3·89	5·05	4·24	0·793	0·201	0·242	0·105	2·48
Difference		−0·10	−0·85	+0·57	−0·25	−0·45	+0·033	+0·006	−0·004	+0·010	+0·46
2	12	12·58	8·58	3·73	4·00	4·03	0·817	0·174	0·236	0·133	3·30
	36	9·24	7·29	4·06	1·95	2·31	0·924	0·179	0·212	0·198	8·57
Difference		−3·34	−1·29	+0·33	−2·05	−1·72	+0·107	+0·005	−0·024	+0·065	+5·27
3	12	12·25	8·85	3·85	3·40	4·26	0·736	0·227	0·234	0·141	3·31
	48	10·00	7·40	4·27	2·60	2·09	1·038	0·199	0·180	0·281	15·44
Difference		−2·25	−1·45	+0·42	−0·80	−2·17	+0·302	−0·028	−0·054	+0·140	+12·13

(The writer is indebted to the Hannah Dairy Research Institute for the supply of the milk.)

It will be seen that in general the results obtained are similar to those of Petersen & Rigor, particularly in the fall of lactose and phosphorus and in the increase in the ash and chlorine. The increase in the protein is not so marked, while a reduction in the fat content, to half its normal value, occurred in the milk of cow No. 2 when she was left unmilked for 36 hours.

In the case of these three cows the chlorine-lactose index is of particular interest. When the milk was left in the udder for 24 hours the index increased slightly but it was still within the normal range, but in milk which was left in the udder for 36 hours the index rose from 3·30 to 8·57, and again when cow No. 3 was left unmilked for 48 hours the index rose to 15·44. The percentage of calcium showed no significant change either for the 24- or 36-hour periods, but an appreciable reduction occurred when the milk was left in the udder for 48 hours.

The intra-mammary pressure was not measured during the experiment nor at the time the foals were weaned.

For the purpose of studying the composition of mare's milk after weaning, samples were obtained from five ponies (ponies 4, 5, 6, 7 and 8) at varying intervals after the foals had been removed. Each sample analysed was obtained after withdrawing as much milk as possible from the udder. The alterations which occurred in the composition were noted in relation to the time from weaning and to the time the milk was allowed to accumulate in the udder. The analytical data are given in Tables XII and XIII. It will be seen that alterations in the composition which occur when milk is allowed to accumulate in the udder for periods longer than would be natural during suckling are in certain respects similar to the changes found to occur in "pressure" milk in the cow. In comparing them, however, it should be realized that while 12 hours is a normal period during which milk is allowed to accumulate in the udder of a cow, it is an abnormally long period for a mare. If a mare whose mammary glands were in active secretion were left unmilked for such a long time the milk would accumulate under considerable pressure.

Protein. Very little alteration was found to occur in the percentage of protein, other than fluctuations within the normal range of variation, until the udder had been left unmilked for 24 hours. In milk allowed to accumulate for 24 hours or longer there was a marked increase in the percentage, over 8 per cent being found on one occasion.

Lactose. The percentage of lactose was not found to decrease until the milk was allowed to accumulate in the udder for 12 hours or longer. With ponies 7 and 8 the greatest volume of milk was obtained at the end of this initial prolonged period, indicating relationship between intra-mammary pressure and low lactose content. After the initial decrease in lactose has occurred, though the percentage may fluctuate it does not return to normal even when the actual volume of milk secreted is small.

A percentage of lactose as low as 1·14 has been found, associated with a chlorine-lactose index of 28·5. The percentage of lactose appears to be inversely proportional to the time since weaning.

Fat. It has previously been found by the writer that there was a decrease in the percentage as the intervals between milkings increased from 2 to 5 hours. In this investigation the percentage was found to be very variable for intervals of 2, 4, 6, 7 and 12 hours, though it was usually low. The most striking change

Table XII. *Percentage composition of mare's milk secreted after weaning the foal* (1933)

Hours after weaning	Interval since milking hours	Total solids	Solids-not-fat	Protein	Fat	Lactose	Chlorine-lactose index	Ash	CaO in the milk %	CaO in the ash %	P_2O_5 in the milk %	P_2O_5 in the ash %	Cl in the milk %	Cl in the ash %	CaO/P_2O_5
Pony 4, foaled 4 May 1933; weaned 19 October 1933															
2	—	11·93	9·43	2·04	2·50	7·15	0·307	0·235	0·080	34·1	0·071	30·2	0·022	9·4	1 : 0·89
8	6	10·59	9·99	2·13	0·60	7·57	0·264	0·292	0·087	29·8	0·089	30·5	0·020	6·9	1 : 1·02
12	4	10·76	9·76	1·85	1·00	7·67	0·326	0·238	0·081	34·1	0·081	34·1	0·025	10·5	1 : 1·00
24	12	9·79	8·89	1·77	0·90	6·81	1·010	0·307	0·093	30·1	0·053	17·3	0·069	22·5	1 : 0·57
31	7	13·20	9·00	2·53	4·20	5·85	1·230	0·322	0·109	33·9	0·047	14·7	0·072	22·4	1 : 0·43
49	18	11·55	9·15	2·10	2·40	6·66	1·396	0·394	0·114	29·0	0·058	14·7	0·093	23·6	1 : 0·51
73	24	12·76	8·36	3·28	4·40	3·57	3·781	0·514	0·123	23·9	0·058	11·3	0·135	26·3	1 : 0·47
97	24	14·32	7·32	4·76	7·00	1·89	9·735	0·671	0·082	12·2	0·035	5·2	0·184	25·0	1 : 0·43
Pony 5, foaled 15 May 1933; weaned 19 October 1933															
2	—	10·81	8·91	1·85	1·90	6·82	0·293	0·243	0·083	34·2	0·092	37·9	0·020	8·2	1 : 1·11
8	6	10·68	8·56	1·05	2·12	7·19	0·264	0·322	0·091	28·3	0·085	26·4	0·019	5·9	1 : 0·93
12	4	10·27	9·52	1·89	0·75	7·34	0·300	0·286	0·088	30·8	0·081	28·3	0·022	7·7	1 : 0·92
24	12	9·12	8·82	1·50	0·30	7·05	0·695	0·267	0·084	31·5	0·058	21·7	0·049	18·4	1 : 0·69
31	7	10·70	9·10	1·68	1·60	7·14	0·672	0·277	0·093	33·6	0·066	23·8	0·048	17·3	1 : 0·71
49	18	7·20	6·20	1·46	1·00	4·22	4·952	0·522	0·068	13·0	0·030	5·8	0·209	40·0	1 : 0·44
73	24	13·92	8·32	2·65	5·60	5·18	2·336	0·490	0·107	21·8	0·043	8·8	0·121	24·7	1 : 0·40
97	24	12·04	6·44	3·41	5·60	2·42	8·181	0·611	0·072	11·8	0·023	3·8	0·198	32·4	1 : 0·32
Pony 6, foaled 22 May 1933; weaned 19 October 1933															
8	—	9·61	9·51	1·89	0·10	7·34	0·258	0·276	0·090	32·7	0·081	29·4	0·019	6·9	1 : 0·90
12	4	9·92	9·49	1·72	0·43	7·50	0·280	0·272	0·081	29·8	0·055	20·2	0·021	7·7	1 : 0·68
24	12	9·03	8·93	1·54	0·10	7·18	0·487	0·213	0·083	39·0	0·061	28·6	0·035	16·4	1 : 0·74
31	7	10·41	9·21	1·75	1·20	7·21	0·651	0·253	0·093	36·8	0·066	26·1	0·047	18·6	1 : 0·71
49	18	10·44	9·79	1·77	0·65	7·68	1·080	0·337	0·092	27·3	0·048	14·2	0·083	24·6	1 : 0·52
73	24	11·81	8·61	2·49	3·20	5·66	2·102	0·460	0·107	23·3	0·055	12·0	0·119	25·9	1 : 0·51
97	24	12·66	5·86	2·85	6·80	2·36	10·042	0·648	0·067	10·3	0·028	4·3	0·237	36·6	1 : 0·42

Table XIII. *Percentage composition of mare's milk secreted after weaning the foal* (1934)

Hours after weaning	Interval since milking hours	Total solids	Solids-not-fat	Protein	Fat	Lactose	Chlorine-lactose index	Freezing-point	*p*H	Volume of milk in the udder in ml.	Ash	CaO in the milk %	CaO in the ash %	P_2O_5 in the milk %	P_2O_5 in the ash %	Cl in the milk %	Cl in the ash %	CaO/P_2O_5
Pony 7, foaled 24 May 1934; weaned 23 October 1934																		
—	—	10·57	9·77	2·16	0·80	7·32	0·287	—	7·14	105	0·291	0·087	29·9	0·066	22·7	0·021	7·2	1 : 0·76
5	—	9·89	9·59	1·91	0·30	7·37	0·366	−0·582°	6·93	310	0·310	0·094	30·3	0·082	26·5	0·027	8·7	1 : 0·87
17	12	8·06	7·96	1·65	0·10	6·92	1·460	−0·575°	7·37	760	0·394	0·098	24·9	0·051	13·0	0·101	25·6	1 : 0·52
41	24	8·20	6·50	4·52	1·70	1·14	28·500	−0·557°	7·30	200	0·844	0·090	10·7	0·046	5·5	0·325	38·5	1 : 0·51
65	24	22·82	13·67	8·16	9·15	4·79	1·524	−0·571°	7·20	110	0·718	0·197	27·4	0·076	10·6	0·073	10·2	1 : 0·39
89	24	10·98	9·08	3·82	1·90	4·72	2·987	−0·570°	7·47	380	0·539	0·106	19·7	0·025	4·6	0·141	26·2	1 : 0·24
137	48	20·38	11·98	7·09	8·40	4·15	4·867	—	7·70	75	0·740	0·040	5·4	—	—	0·202	27·3	—
Pony 8, foaled 4 May 1934; weaned 28 October 1934																		
—	—	10·18	9·57	2·10	0·61	7·16	0·363	−0·559°	7·10	160	0·315	0·096	30·5	0·090	28·6	0·026	8·3	1 : 0·94
5	—	9·50	9·20	1·64	0·30	7·28	0·274	−0·582°	7·06	480	0·285	0·084	29·5	0·083	29·1	0·020	7·0	1 : 0·99
17	12	7·43	7·23	1·48	0·20	5·26	3·080	−0·579°	7·50	940	0·491	0·094	19·1	0·046	9·4	0·162	33·0	1 : 0·49
41	24	17·08	8·58	5·82	8·50	2·01	13·830	−0·597°	7·37	180	0·754	0·105	13·9	0·051	6·8	0·278	36·9	1 : 0·49
65	24	—	—	—	Sample lost		—	—	—	310	—	—	—	Sample lost	—	—	—	—
89	24	12·86	8·56	3·29	4·30	4·78	3·033	−0·588°	7·40	500	0·492	0·076	15·5	0·031	6·3	0·145	29·5	1 : 0·41
137	48	12·11	7·31	3·60	4·80	3·02	7·649	−0·571°	7·66	275	0·687	0·041	6·0	0·020	2·9	0·231	33·6	1 : 0·49

is the *increase* in the percentage (which on one occasion reached 9·15), that occurred in the milk of all the five ponies after it had been left in the udder for 24 hours or longer.

These *increases* accompany increases in the percentages of protein, whereas in the case of cows, *decreases* in fat appear to accompany increases in protein when the milk is allowed to accumulate in the udder for long periods.

Ash. The percentage of ash has been found to increase, with some fluctuation, from the time the foal is removed from the mare to the cessation of secretion. These increases are roughly proportional to decreases in the percentage of lactose: the maximum ash content was invariably found to be associated with the minimum lactose and with a high chlorine-lactose index. The highest ash content coincided with the highest "index" and, on all but one occasion, with the highest chlorine content. The percentage of ash in the post-weaning milk may be as high as two-and-a-half or even three times its normal value.

Chlorine. The percentage of chlorine increases steadily in the milk during this period, and at the end of lactation may be ten times the preweaning value. The percentage in the ash increases to approximately five times its normal value.

The extent to which either the percentage of chlorine and lactose or the chlorine-lactose index deviates from the normal indicates the extent to which the milk has changed in character. The chlorine-lactose index was in every case found to be greater than normal when the milk was allowed to accumulate in the udder for 12 hours or longer.

Calcium. The percentage of calcium in the post-weaning milk fluctuates but shows a tendency to increase. However, in the final samples the value was less than the normal for the end of lactation, in four of the five milks analysed. This reduction was most marked in the milk of ponies 7 and 8 when the udders were emptied 137 hours after weaning, and milking had been delayed for 48 hours, the percentages falling from 0·087 to 0·040 and from 0·096 to 0·041. The percentage of calcium in the ash fell owing to an increase in the percentage of the total ash.

Phosphorus. A considerable decrease has been found in the percentage of phosphorus, both in the milk and in the ash, the greatest reduction occurring in the final milkings. As the calcium does not decrease similarly, the ratio CaO/P_2O_5 is narrowed.

The changes which occur in the composition of the milk when its removal is delayed are more pronounced when cessation of milking is more sudden than when the udder is emptied at shorter intervals after weaning. In the case of ponies 7 and 8 the udder was emptied 5 hours after weaning (the time of final sucking by the foal being unknown), again after 12 hours, three times at 24-hour intervals and finally when the milk had been left in the udder for 48 hours. Marked changes in the composition occurred at the end of the 12-hour period and still greater changes at the end of the longer periods. In ponies 4, 5 and 6,

which were milked dry at shorter and more frequent intervals, alterations in the composition are not so pronounced.

Hydrogen-ion concentration. In the milk withdrawn from the ponies after weaning (the Clydesdale's post-weaning milk was not available), the *p*H rose, the highest values being 7·70 for pony 7 and 7·66 for pony 8 when the milk was left in the udder for 48 hours. The normal *p*H for horse's serum is 7·34 at body temperature and 7·4 at 20° C. Petersen & Rigor, who determined the *p*H of cow's milk electrometrically when milking was delayed for 24, 36 and 120 hours, found it to rise from 6·4 to 7·3 at the end of the 120-hour period. They remarked that the *p*H of "pressure" milk is higher than that of the serum of the cow.

Freezing-point. As is shown in Table XIII the freezing-point in the post-weaning milk varied from −0·557 to −0·582° for pony 7 (five records) and from −0·559 to −0·597° for pony 8 (six records).

CONCLUSION

(*a*) It has been shown that the percentage composition of mare's milk changes in a definite manner during the course of lactation. It is therefore important not only to know the percentage composition of a sample of milk but the period of lactation at which it was withdrawn, before any conclusion as to its normality is reached. Random samples are therefore of little use *per se*. The dates of parturition must be known, and no one sample of mare's milk can be said to be representative.

(*b*) When cow's milk is left in the udder for periods sufficient to induce intra-mammary pressure, its composition is so changed that it may contain less than 8·5 per cent solids-not-fat and less than 3 per cent of butter-fat, the lowest legal quantities for normal milk. Similar changes have been found in the milk of the mare, following weaning.

SUMMARY

1. The investigation was for the purpose of ascertaining the cause of the variations previously noted by the author as occurring in mare's milk.

2. Determinations have been made of the composition of the milk of eight Shetland ponies and one Clydesdale mare throughout the lactation period, with observations on the milk of numerous thoroughbred mares. The constituents estimated were the total solids, solids-not-fat, protein, fat, lactose, total ash, CaO, P_2O_5, and chlorine. The CaO, P_2O_5 and chlorine percentages in the ash of the milk were also determined. The freezing-point and *p*H were recorded in a number of samples. The animals were kept under natural conditions at grass.

3. It was found that as lactation advanced the percentage of total solids, lactose and fat of the milk increased in a linear manner, whereas the percentage of solids-not-fat, protein, CaO, and P_2O_5 decreased linearly.

4. The regression of the total solids and the chlorine was parabolic in character as also was that of the chlorine-lactose index.

5. The CaO and P_2O_5 decreased in close conformity with one another, the CaO/P_2O_5 ratio remaining constant throughout the period of lactation.

6. The conclusion is drawn that "stage of lactation" is the principal factor governing variation in the percentage composition of mare's milk.

7. It has been shown that intra-mammary pressure brought about by sudden cessation of natural removal of the milk by the offspring or in the case of the cow by hand-milking is also a very important factor in changing the composition of milk.

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156. THE COMPOSITION OF MILK AND WHEY, WITH SPECIAL REFERENCE TO THE PARTITION OF CALCIUM AND PHOSPHORUS

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(With 15 Figures)

The discovery by Pyne (1) that the colloidal phosphate of milk is largely, if not entirely, tricalcium phosphate was a valuable link in the chain of evidence required for the complete elucidation of the mineral equilibria of milk. A method for estimating tricalcium phosphate in milk was examined by the writer (2) in a previous paper, and the suggestion was made that it should prove an effective means of exploring the hitherto obscure partition of calcium and phosphorus. The present paper gives the results of work undertaken with this end in view.

Fifty-four samples of mixed milk (alternatively a.m. and p.m.), from the College herd of thirty-six to forty-eight Dairy Shorthorns, were taken at regular intervals during the period October 1935 to November 1936. All samples were kept in cold storage at 45° F. until required in the laboratory.

Methods of analysis

On arrival in the laboratory, the samples were placed in a water-bath at 30° C. and then cooled to room temperature. Quantities required for the various determinations were measured with a pipette, and the weight of milk or whey so discharged was subsequently ascertained. All estimations were made in duplicate.

Soluble constituents

For this purpose the analyses were conducted on the rennet whey, correcting for the removal of fat and protein by the use of the factor

$$\frac{100-(F+P-0{\cdot}4)}{100},$$

where F = percentage fat, and P = percentage nitrogen × 6·38. This factor has been found suitable for converting whey percentages to soluble milk constituents (2).

The whey was prepared by adding 225 ml. of milk at 30° C. to 1·5 ml. of rennet extract, maintaining at 30° C. in a water-bath, cutting with a stainless steel knife when of the requisite firmness, and then filtering through a battery of four Whatman No. 40 11 cm. filter papers. The time of coagulation was

Table I

	A.M. samples		P.M. samples		All samples	
	Mean	S.D. ±	Mean	S.D. ±	Mean	S.D. ±
Rennet coagulation (min.)	2·00	0·22	2·45	0·35	2·22	0·36
% fat	3·64	0·20	3·87	0·21	3·75	0·24
% solids-not-fat	8·86	0·14	8·90	0·13	8·88	0·14
% ash	0·766	0·017	0·768	0·012	0·767	0·015
% total protein:						
Kjeldahl	3·44	0·19	3·42	0·18	3·43	0·18
Pyne's method	—	—	—	—	3·41	0·15
% caseinogen	2·67	0·19	2·65	0·16	2·66	0·17
% soluble N × 6·38	0·77	0·13	0·77	0·09	0·77	0·11
Titration values, ml. *N*/10 alkali per 10 ml. sample						
M_1	2·20	0·08	2·18	0·08	2·19	0·08
M_2	0·79	0·09	0·77	0·08	0·78	0·08
W_1	1·41	0·09	1·40	0·07	1·41	0·08
W_2	1·01	0·06	0·99	0·06	1·00	0·06
Titration factors per 10 g. milk (ml. *N*/10 alkali)						
M_1	2·14	0·08	2·13	0·08	2·14	0·08
Caseinogen acidity	0·86	0·05	0·85	0·05	0·86	0·05
Serum acidity	0·91	0·06	0·90	0·05	0·91	0·06
Overrun	0·37	0·03	0·38	0·03	0·37	0·03
Caseinogen acidity per gram caseinogen	0·32	0·03	0·32	0·03	0·32	0·02
% tricalcium phosphate	0·1549	0·0104	0·1559	0·0112	0·1554	0·0109
Calcium distribution (% CaO)						
Total	0·1796	0·0128	0·1836	0·0103	0·1816	0·0118
Soluble	0·0591	0·0049	0·0618	0·0046	0·0604	0·0049
As $Ca_3P_2O_8$	0·0840	0·0056	0·0845	0·0061	0·0843	0·0059
Caseinogen CaO	0·0365	0·0078	0·0373	0·0073	0·0369	0·0076
Calcium distribution. Ratios to total = 100						
Soluble	32·91	1·80	33·66	2·00	33·26	1·94
As $Ca_3P_2O_8$	46·77	3·52	46·02	3·33	46·42	3·46
Caseinogen CaO	20·32	3·70	20·32	3·61	20·32	3·66
Calcium distribution per gram caseinogen						
Caseinogen CaO	0·0137	0·0027	0·0141	0·0030	0·0139	0·0028
CaO as $Ca_3P_2O_8$	0·0315	0·0024	0·0319	0·0017	0·0317	0·0021
Ratio CaO as tricalcium phosphate/caseinogen CaO	2·30	1·039	2·27	0·87	2·28	0·96
Phosphorus distribution (% P_2O_5)						
Total	0·2540	0·0127	0·2602	0·0185	0·2571	0·0161
Soluble	0·1285	0·0080	0·1343	0·0128	0·1315	0·0111
Inorganic	0·1634	0·0063	0·1631	0·0071	0·1632	0·0067
Organic	0·0906	0·0120	0·0971	0·0168	0·0939	0·0152
Ratio to total = 100						
Soluble	50·59	2·37	51·61	2·13	51·15	2·32
Insoluble	49·41	—	48·39	—	48·85	—
Inorganic	64·33	3·57	62·68	3·83	63·48	3·85
Organic	35·67	—	37·32	—	36·52	—
Inorganic P_2O_5						
Soluble	0·0864	0·0048	0·0857	0·0037	0·0861	0·0044
Insoluble	0·0770	0·0058	0·0774	0·0053	0·0771	0·0056
As $Ca_3P_2O_8$	0·0709	0·0048	0·0714	0·0050	0·0712	0·0050
Ratio to total inorganic = 100						
Soluble	52·88	2·33	52·55	2·36	52·76	2·35
Insoluble	47·12	—	47·45	—	47·24	—
Organic P_2O_5						
Soluble	0·0421	0·0080	0·0486	0·0125	0·0454	0·0110
Insoluble	0·0485	0·0079	0·0485	0·0076	0·0485	0·0078

Table I (*continued*)

	A.M. samples		P.M. samples		All samples	
	Mean	S.D. ±	Mean	S.D. ±	Mean	S.D. ±
	Ratio to total organic = 100					
Soluble	46·47	5·48	50·05	5·83	48·35	5·86
Insoluble	53·53	—	49·95	—	51·65	—
	Composition of whey (percentages by weight)					
CaO	0·0631	0·0052	0·0664	0·0048	0·0647	0·0053
Total P_2O_5	0·1369	0·0083	0·1441	0·0128	0·1405	0·0115
Inorganic P_2O_5	0·0927	0·0052	0·0913	0·0041	0·0920	0·0047
Organic P_2O_5	0·0042	0·0078	0·0528	0·0137	0·0485	0·0118
Nitrogen (Kjeldahl)	0·1629	0·0131	0·1663	0·0100	0·1646	0·0118

determined from the time of mixing to the first appearance of solid flecks of curd on the sides of the beaker, which was rocked gently during the test.

Fat was estimated by the Gerber method, and *solids-not-fat* by the Richmond Formula.

Tricalcium phosphate. The method is fully described in the previous paper (2), but since it is of fundamental importance to the present work, it is restated. 10 ml. portions of milk and whey were titrated with $N/10$ alkali, using 6 drops of 0·5 per cent phenolphthalein as indicator. The titration was repeated on further 10 ml. portions after addition of 0·4 ml. saturated potassium oxalate solution (neutral), and allowing to stand for 2 min. With the milk titrations it was found necessary to use a colour standard in order to ensure conformity of results from week to week. The standard selected was a 0·25 per cent aqueous solution of rosaniline hydrochloride, added at the rate of 0·14 ml. per 25 ml. milk. This was in excess of the depth of colour usually adopted for acidity tests, as it was essential to ensure that the titrations should end as near as possible to the monohydrogen phosphate stage. All figures so obtained were converted to a percentage milk basis.

Potassium oxalate has a dual influence on the titration of milk. It eliminates the slow precipitation of tricalcium phosphate and simultaneous liberation of phosphoric acid which are known to occur during the titration, and thus removes a factor which, for the sake of clarity, will be termed the overrun (*o*). These changes are represented by Van Slyke and Bosworth (3) according to the following equations:

$$\begin{aligned} &CaHPO_4 + 2H_2O = Ca(OH)_2 + H_3PO_4, \\ &2CaHPO_4 + Ca(OH)_2 = Ca_3P_2O_8 + 2H_2O \\ \hline &3CaHPO_4 = Ca_3P_2O_8 + H_3PO_4 \end{aligned},$$

By precipitation of calcium as oxalate these changes are prevented, since acid potassium phosphate is only monobasic to phenolphthalein. Further, potassium oxalate reacts with tri-calcium phosphate to produce alkaline potassium phosphate:

$$Ca_3P_2O_8 + 3K_2 \text{ oxalate} = 3Ca \text{ oxalate} + 2K_3PO_4.$$

If M_1 = titre as $N/10$ alkali per 10 g. milk, W_1 = titre as $N/10$ alkali per 10 g. whey (times whey factor), and M_2 and W_2 are the corresponding values after addition of oxalate; then for fresh milk we have:

M_1 = caseinogen acidity (c) + serum acidity (s) + overrun (o);
$M_2 = c + s$ − alkalinity produced from $Ca_3P_2O_8$;
$W_1 = s + o$;
$W_2 = s$;
$M_1 - W_1 = c$ and $W_1 - W_2 = o$.

Thus the alkalinity equivalent to tricalcium phosphate is obtained as

$$(M_1 - M_2) - (W_1 - W_2).$$

Total protein was determined by the Kjeldahl method, using the factor percentage N×6·38. For the purpose of comparison, the modified aldehyde figure method of Pyne (4) was also used.

Caseinogen was estimated by Moir's method (5).

Total P_2O_5 was estimated on 10 ml. portions of milk or whey, using the nitric-sulphuric digestion method, followed by the gravimetric molybdate-magnesia procedure.

Inorganic P_2O_5 was determined on the trichloracetic acid filtrates by the colorimetric method previously described (2).

Total and soluble CaO were also determined on the trichloracetic acid filtrates by precipitation as oxalate after neutralization and reacidification with dilute acetic acid.

Ash was determined on 10 ml. portions by incineration at dull red heat.

Values obtained by difference:

Inorganic colloidal P_2O_5	Total − soluble
Colloidal CaO	Total − soluble
Caseinogen CaO	Colloidal − tricalcium phosphate CaO
Soluble protein	Total − caseinogen
Total organic P_2O_5	Total − inorganic
Soluble organic P_2O_5	Total soluble − inorganic soluble
Insoluble organic P_2O_5	Total organic − soluble organic.

The terms soluble and insoluble refer to the state of the various constituents as they exist in milk and are not comparable to the "acid-soluble" determinations used in other biochemical works.

Mean values

Table I presents the mean values and standard deviations of all estimations for morning, evening, and mixed milk, and the corresponding wheys.

DISCUSSION

No outstanding differences are apparent in the results of morning and evening samples, except in the time of rennet coagulation, where a difference of 0·45 min. is observed. This difference is significant, since the determination was capable of an accuracy of ± 0·1 min. As there was nothing in the a.m. and p.m. figures to throw light on this difference, the explanation was sought elsewhere. At the time of the tests, although the morning samples were only 3–4 hours old, the evening ones had been kept for some 15–16 hours. It therefore seemed likely that prolonged storage at 45° F. in the case of the evening's milk might account for the relatively longer time of coagulation of these samples. To check this hypothesis, rennet tests were conducted on samples of mixed milk kept in cold store for varying lengths of time. From the results of these experiments, shown in Table II, it is clear that the observed differences in a.m. and p.m. rennet coagulation may be attributed to the effect of low-temperature storage.

Table II

Sample	A		B		C		D		E			F			G	
Age in hours	5	12	3	27	3	10	3	27	3	10	27	4	10	28	3	27
Rennet time in min.	4·3	5·0	3·4	3·7	2·8	3·1	1·9	2·4	2·1	2·4	2·4	2·5	2·8	2·9	3·3	3·6

It is interesting to note that Mattick & Hallett (6) observed a similar retardation up to 5 hours after milk had been subjected to heat treatment, while Powell & Palmer (7) found a similar hysteresis effect on heated colloidal calcium caseinogenate-calcium phosphate systems.

The means for *fat, solids-not-fat, and ash* do not differ greatly from those obtained by other workers (8), and need no comment.

Total protein. The agreement between the Kjeldahl and Pyne's amended aldehyde figure methods is very striking, and in view of its greater convenience and expedience the latter should prove a valuable routine test.

Caseinogen. The mean 2·66 represents 77·6 per cent of total nitrogen. Davies (9) quotes 2·45 and 2·52 per cent for Dairy Shorthorns, representing 76·7 and 76·5 per cent of total nitrogen respectively.

Titration values. While the mean M_1 (2·14) is in excess of the titration value normally accepted for fresh milk, for reasons already explained the ratio of its component factors will not be affected thereby. Thus if

$$M_1 = c + s + o,$$
$$2{\cdot}14 = 0{\cdot}86 + 0{\cdot}91 + 0{\cdot}37, \text{ or}$$
$$1 = 0{\cdot}40 + 0{\cdot}42 + 0{\cdot}18;$$

that is, approximately four-fifths the titratable acidity of fresh milk is shared equally by caseinogen and serum acidity, while the remaining one-fifth is due to the overrun.

Caseinogen CaO. The writer has previously observed that when milk is allowed to develop acidity up to coagulation point, the increase in caseinogen

acidity is directly equivalent to the CaO detached from this protein (2). The mean of thirty-four samples gave an increase of caseinogen acidity corresponding to 0·0364 per cent CaO, while in the present series 0·0369 per cent is obtained. Lehman (11) gives 1·45 per cent CaO per 100 g. caseinogen, Soldner (12) 1·55 per cent, and Richmond (13) 1·4 per cent corresponding to a formula Ca_3Na_3H caseinogen.$2Ca_3P_2O_8$. Although the figure 1·39 per cent obtained in the present investigation agrees with Richmond's value, the acidity of the complex is much in excess of that indicated by his formula.

The acidity per gram caseinogen (0·32 ml. *N* alkali) is somewhat greater than that found by Palmer & Richardson (23) (0·27 ml.). Adding to this figure, the acid equivalent of 0·0139 g. CaO attached to the protein (0·50 ml. *N* acid), the total 0·82 is obtained, a value which would account for almost all the base-binding properties of caseinogen as found by other workers (0·90) (10). It appears, therefore, that this protein is slightly more than half saturated with calcium, but the standard deviations from the mean show that this is subject to considerable variation.

Tricalcium phosphate. The mean of the present series (0·1554 per cent) agrees with that previously obtained from thirty-three samples of mixed milk (0·1538 per cent) (2), and alone accounts for nearly half of the total inorganic phosphate and total calcium respectively.

The ratio of calcium as tricalcium phosphate to caseinogen calcium is of special interest in view of the theory of Pyne (1), that the colloidal phosphate may be attached to the caseinogen molecule through the medium of the calcium attached to the protein:

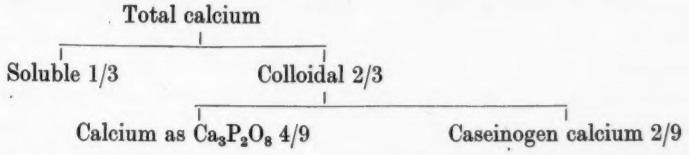
$$(\text{Protein}) = (COO)_2Ca + Ca_3P_2O_8 = (\text{Protein}) = (COO—Ca—PO_4{=}Ca)_2.$$

If the phosphate is thus chemically bound to caseinogen, one would expect this ratio to be 3/1. Further, one would not expect to find any great variation from this figure. The mean value is 2·28 and is obtained from figures varying from 1·5 to 6·3. On five occasions out of fifty-two, the ratio exceeded 3/1. The seasonal graphs show that when the total calcium falls sharply its effect is felt more by caseinogen calcium than by tricalcium phosphate. Thus it would appear that the formation of tricalcium phosphate takes precedence over the saturation of caseinogen by calcium. It is difficult to reconcile these facts with the theory of chemical attachment of tricalcium phosphate to caseinogen. Although the mean ratio approximates to the 2/1 figure of Richmond's formula, it is apparent that no chemical formula can adequately depict the conditions controlling this colloidal complex.

The calcium partition. The mean percentage total calcium agrees with the values of Golding *et al.* (14), Cranfield *et al.* (15), and Sommer & Hart (16). Soluble calcium, representing 33·26 per cent of total, is in agreement with the 32·96 per cent obtained by Van Slyke & Bosworth (17), although these workers used a Pasteur-Chamberland candle for preparing the serum. Considerable variation, however, was observed (29–37 per cent), although the limits were not so

extreme as those experienced by these workers (23–46 per cent). Of the colloidal calcium, representing approximately two-thirds the total, caseinogen calcium shows the greater variation.

The partition of calcium may be represented approximately as



Total P_2O_5. The mean value (0·257), while higher than those of Golding *et al.* (14) and Cranfield *et al.* (15), is in agreement with those of Meigs *et al.* (18) and Sommer & Hart (16).

Soluble P_2O_5, representing 51·5 per cent of total, is considerably greater than that obtained by Van Slyke & Bosworth (17) (44 per cent); and again, the extremes noted by these workers (27–64 per cent) were not experienced in this work (47–57 per cent). The difference may be due, in part, to the two different methods of preparing the sera.

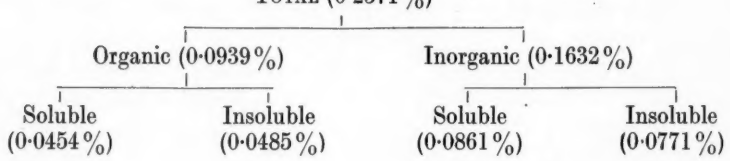
Ratio inorganic P_2O_5 *to total* P_2O_5. The mean, 63·5 per cent, is lower than that determined by Jordan *et al.* (19), whose values ranged from 65 to 80 per cent. Lenstrup (20), using picric acid as precipitant, found the mean ratio for fifteen samples to be 70 per cent. Sanders (21), using trichloracetic acid as precipitant, found 75 per cent, but this figure was shown to be high by Graham & Kay (22), on account of the inclusion of acid-soluble organic phosphorus which Graham assumed to be absent. The figures obtained by these two workers were 64·6 per cent for Ayrshires, 67·5 per cent for Holstein-Friesians and 63·7 per cent for Jerseys. The mean of the present series is therefore in close agreement with those of Graham & Kay. The high values found by other workers must be attributed at least in part to failure of the analytical methods in discriminating between organic and inorganic phosphorus.

Of the *total inorganic phosphorus*, 52·8 per cent is soluble. The mean value for insoluble inorganic P_2O_5, 0·0771 per cent, differs from the P_2O_5 in tricalcium phosphate, 0·0712 per cent, by 0·0059. This difference was positive in fifty-two cases out of the fifty-four examined. If one concedes an accuracy in the titration estimation of tricalcium phosphate of $\pm 0{\cdot}05$ ml. alkali, the experimental variation in the percentage of P_2O_5 contained therein would be $\pm 0{\cdot}0036$. From its constancy of sign, the writer is of the opinion that the observed mean difference is significant and due probably to the presence of colloidal magnesium phosphate. Estimations of magnesium in milk and whey of nine samples gave 0·0150 per cent total, 0·0107 per cent soluble, leaving 0·0043 per cent colloidal Mg of which some, at least, will be in the form of phosphate.

The *organic phosphorus* is resolved into 48·4 per cent soluble and 51·6 per cent insoluble. Taking the mean 0·0485 per cent insoluble organic P_2O_5, and

deducting 0·0426 as the P_2O_5 content of 2·66 per cent caseinogen, the value 0·0059 per cent is obtained, representing phospholipid P_2O_5. Sanders (21) considers the average phospholipid content of milk to be 0·07 per cent containing 0·0062 per cent P_2O_5, but Graham & Kay (22) estimate at least 0·0107 per cent P_2O_5 present in this form. Again, Holm *et al.* (24) find 0·0030 per cent, while Mohr *et al.* (25) record 0·0034 per cent P_2O_5 in the lipid form. This lack of conformity in published data may be due to the difficulties encountered in the separation of phospholipids and to the variable composition of these compounds. The value 0·0059 per cent P_2O_5 calculated as lipid P_2O_5 from the figures obtained in the present investigation is subject to the inaccuracies of all difference figures, and is based on the assumption that only negligible amounts of phospholipids escape in the whey. The mean percentage soluble organic P_2O_5 (0·0454) is greater than that found by Graham & Kay (22) (0·0227), who found great variation in the samples from the six cows of each group under observation. Even when a larger number of samples of mixed milk are examined over a longer period, it is evident from the standard deviations that both soluble and insoluble organic P_2O_5 are subject to great variation as compared with their inorganic counterparts.

The partition of milk P_2O_5 may be summarized as follows:



Seasonal variation

The observed seasonal changes in the main constituents and ratios are shown in Figs. 2–13, while in Table III and Fig. 1 are presented the lactation composition of the herd over the period under observation. In Table IV are shown the maximum screen air temperatures (monthly means) as recorded by the College meteorological station.

Rennet coagulation. The fluctuating nature of the seasonal curve (Fig. 2*a*) is at once apparent and has also been observed by Golding *et al.* (26). These workers record a quickening effect due to the introduction of newly calved cows, but in the present work there appears to be a certain amount of lag, until the period of maximum yield is reached. The retarding influence operating in the spring and summer months was also observed by these workers, although in one season the effect was reversed (14).

Fat. Although the general trend of this curve (Fig. 2*b*) agrees with the findings of Baker & Cranfield (27) and other workers, its similarity to the lactation graph showing the percentage of cows in advanced lactation is very striking. So also is its inverse relationship to the average yield per cow per

Table III. *Monthly lactation composition of the herd*

Days in milk	0–50	50–100	100–150	150–200	200–250	250–300	300–350	350–400	400–450	450–500	500–550	550–600
1935	Percentage of cows											
Sept.	4·4	13·3	11·1	24·5	20·0	13·3	4·5	6·7	2·2	—	—	—
Oct.	4·9	9·3	9·8	21·3	23·1	16·9	4·9	8·5	1·3	—	—	—
Nov.	12·3	3·7	11·2	11·8	23·5	17·7	15·0	1·6	3·2	—	—	—
Dec.	13·5	5·7	7·9	9·3	18·6	22·8	14·3	3·6	4·3	—	—	—
1936												
Jan.	13·1	13·9	2·9	7·3	10·9	23·4	15·3	8·8	0·0	4·4	—	—
Feb.	12·1	14·3	10·5	5·0	9·9	12·7	18·8	10·0	2·2	2·8	1·7	—
March	20·5	16·9	13·9	4·8	6·0	9·0	10·9	9·0	4·2	0·0	4·8	—
April	28·4	17·6	13·5	16·2	4·1	4·0	6·8	2·7	2·7	2·7	0·0	1·3
May	21·4	22·0	15·9	15·2	9·7	6·2	1·4	2·7	2·8	2·7	0·0	0·0
June	28·7	17·7	17·1	14·0	13·4	4·9	1·2	0·6	0·0	0·0	2·4	0·0
July	20·2	21·0	19·9	13·9	12·4	11·2	0·7	0·0	0·0	0·0	0·7	0·0
Aug.	16·3	20·9	17·1	18·0	13·0	10·9	3·8	0·0	0·0	0·0	0·0	0·0
Sept.	8·8	14·3	25·2	17·6	15·4	12·1	6·6	0·0	0·0	0·0	0·0	0·0
Oct.	7·0	12·7	24·9	15·0	17·9	12·7	7·5	2·3	0·0	0·0	0·0	0·0
Nov.	11·5	10·8	15·4	26·9	15·4	13·9	3·8	2·3	0·0	0·0	0·0	0·0

Table IV. *Maximum screen temperatures. Monthly means,* °F.

1935			1936									
Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sep.	Oct.	Nov.
56·3	49·5	42·3	42·3	41·5	49·5	50·1	61·5	61·1	66·4	69·7	66·5	56·4	48·7

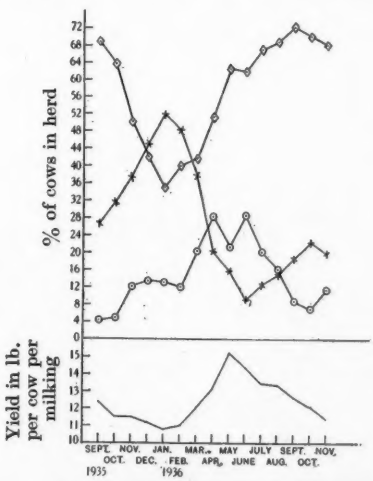


Fig. 1. Lactation composition of herd.

% of cows 0–50 days in milk
% of cows 50–250 days in milk
% of cows over 250 days in milk

milking and the percentage of cows in early lactation. These factors are so obviously connected that in the present investigation, it seems that the effects of season and commencement of pasture feeding in May are masked by the lactation composition of the herd.

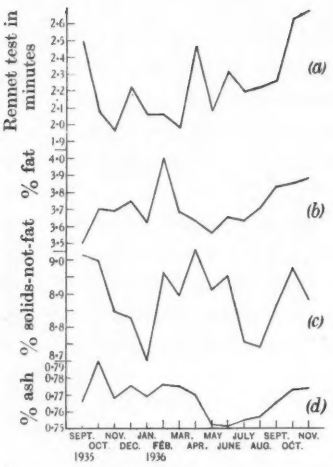


Fig. 2.

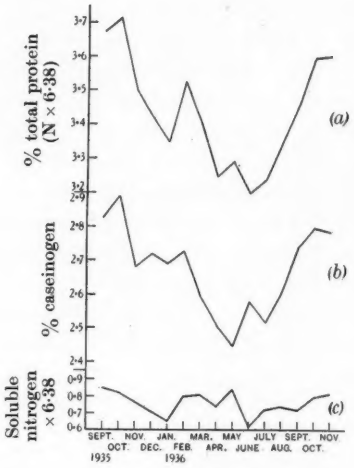


Fig. 3.

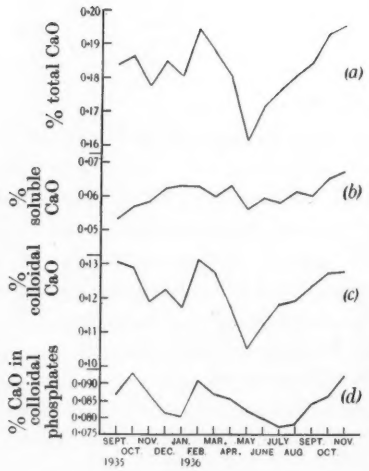


Fig. 4.

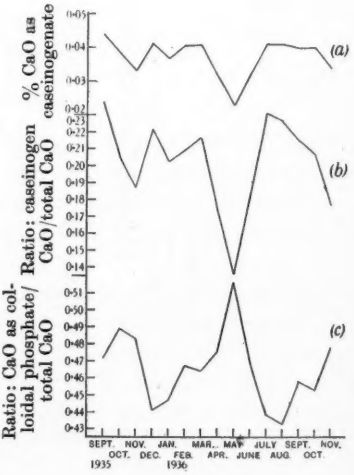


Fig. 5.

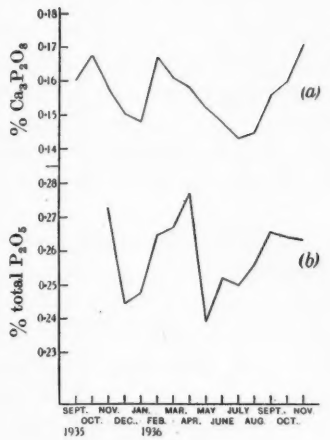


Fig. 6.

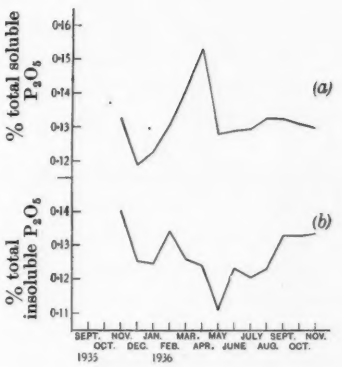


Fig. 7.

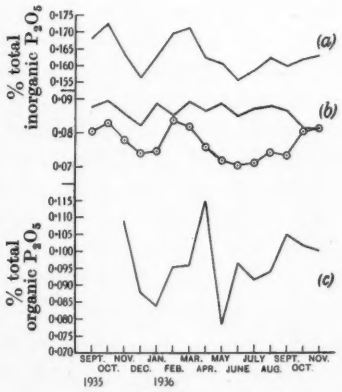


Fig. 8.

% soluble inorganic P_2O_5
% insoluble inorganic P_2O_5

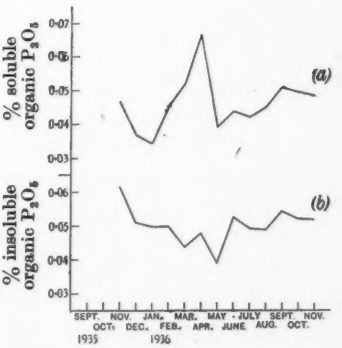


Fig. 9.

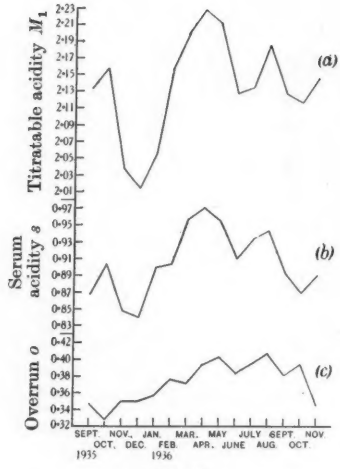


Fig. 10.

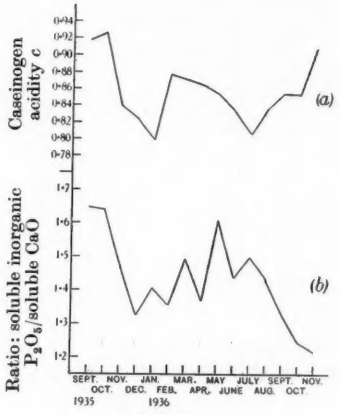


Fig. 11.

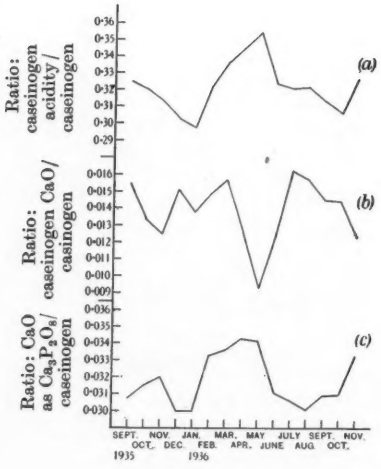


Fig. 12.

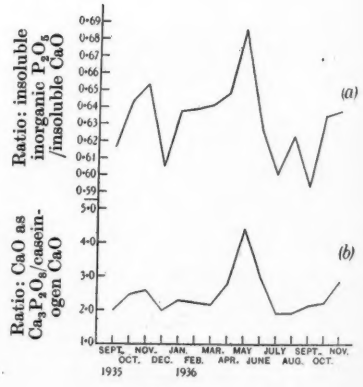

Ratio: insoluble inorganic P_2O_5/insoluble CaO
Ratio: CaO as $Ca_3P_2O_8$/caseinogen CaO
(a)
(b)
SEPT. NOV. JAN. MAR. MAY JULY SEPT. NOV.
OCT. DEC. FEB. APR. JUNE AUG. OCT.
1935 1936

Fig. 13.

Solids-not-fat. The pronounced fall in solids-not-fat in late summer (Fig. 2*c*) has been observed by Richmond (28) and to a lesser degree by Baker & Cranfield (27), but the corresponding fall in January is abnormal. This decline coincides with the maximum percentage of cows in advanced lactation, eight of which were barren. The individual solids-not-fat content of the milk of these eight cows, representing 18 per cent of the herd, cannot be given; so that it is impossible to calculate their effect on the mixed milk. Since barren cows in advanced lactation are known to give lower solids-not-fat than normal (29), this must be regarded as a factor contributing to the abnormal decline in January. Six of these cows were taken from the herd in April and the two remainders in July.

Ash. From April to September, the ash is low, mainly on account of depressions in total CaO and insoluble inorganic P_2O_5.

Protein (Fig. 3). A very marked decline is observed in early spring, which is not recovered until the following autumn—an effect shared equally by total protein and caseinogen. It is significant that the lowest values of caseinogen occur in May at the beginning of the grazing season, in view of the findings of Golding *et al.* (14) that substantial increases of total protein and caseinogen occur in this month. The rise in autumn was observed by these workers. It therefore appears that the minima observed in May are probably related to the high proportion of cows in early lactation rather than to seasonal influences.

Total CaO (Fig. 4*a*). A distinct increase from January to February, followed by a steady decrease to May, and a subsequent recovery characterize the total CaO graph. The sharp increase which precedes the spring and summer decline was also observed by Golding *et al.* (26), and, although occurring in January–February in the 1932 series, in 1933 it was observed in April. In the course of work in 1935, to which reference has already been made, the writer noticed this increase in total calcium occurring in the February of that year. In none of these instances can the effect be attributed to the commencement of the grazing season. Fig. 4*d* shows that the increase is borne mostly by tricalcium phosphate. The minimum values of total CaO which are seen to operate in May are in sharp contrast with the observations of Davies & Provan (30), and Hess *et al.* (31) who find that increases occur at the commencement of pasture feeding. Cranfield *et al.* (15), however, observed a depression in May and minimum values in August. In the present work, the low values observed in May are at least partly the result of the high percentage of cows in early lactation, as it is known that milk from such animals may be low in total calcium content (16, 18, 32, 33, 34). Further, the proportion of cows in advanced lactation, whose milk would be relatively high in total CaO, shows an abrupt decline in this period, in fact the peak and minimum of the CaO graph almost coincide with those in the graph showing the percentage of cows in advanced lactation.

Soluble CaO (Fig. 4*b*). Except for a marked rise in April, the graph follows the general trend of total CaO.

Colloidal CaO (Fig. 4*c*) shows an abrupt decline in May, which is due mostly to the extremely low values for *caseinogen* CaO (Fig. 5*a*). As the caseinogen itself reaches a minimum at the same time, one would expect to find low values for caseinogen CaO; but Fig. 12*b* shows that the degree of saturation per gram caseinogen is lowest in this month.

Tricalcium phosphate (Fig. 6*a*). The seasonal graph is characterized by an abrupt increase from January to February, which almost entirely accounts for the similar increase in total CaO, a steady decline until August and a subsequent recovery.

The differences in the partition of calcium are emphasized in the ratios to total calcium (Figs. 5*b* and *c*).

Total P_2O_5 (Fig. 6*b*). Marked variation is observed in total P_2O_5, rising sharply from December to April, then showing an abrupt decline to a minimum in May, followed by a steady increase to autumn. Golding *et al.*(26) found no conformity in seasonal observations of total P_2O_5, and suggested that such variations as were observed were the result of several factors. The effect of lactation on total phosphorus is by no means well established, the published data showing a serious lack of agreement. This lack of conformity is, in the writer's opinion, due mostly to the fact that total phosphorus is the sum of four independent factors, soluble and insoluble, organic and inorganic. Thus, the peak of the total P_2O_5 graph in April is not observed with *insoluble* P_2O_5 (Fig. 7*b*), being entirely due to *soluble* P_2O_5 (Fig. 7*a*).

Total inorganic P_2O_5 (Fig. 8*a*), *soluble inorganic* P_2O_5 (Fig. 8*b*), *insoluble inorganic* P_2O_5 (Fig. 8*b*). Apart from a decline in Autumn–December the soluble inorganic P_2O_5, while fluctuating somewhat, does not appear to be greatly affected by seasonal changes; so that the total inorganic P_2O_5 curve is dominated by insoluble inorganic P_2O_5, which exists mainly as $Ca_3P_2O_8$. While Davies & Provan(30) record an increase in inorganic P_2O_5 as the result of grazing, Lenstrup(20) finds the contrary effect. Fig. 8*a* adds support to the latter conclusion.

Total organic P_2O_5 (Fig. 8*c*), *soluble organic* P_2O_5 (Fig. 9*a*), *insoluble organic* P_2O_5 (Fig. 9*b*). Total organic phosphorus is subject to great variation, due mainly to the soluble fraction which shows a very sharp rise from January to April with a rapid fall to May, followed by a steady recovery. These results are at variance with the view of Lenstrup(20), that only inorganic P_2O_5 shows marked seasonal variation.

Titration values (Figs. 10 and 11*a*). The most prominent features of Fig. 10*a* are the abrupt decline to December, the pronounced rise to April, and the decline which follows. Golding *et al.*(26) also observed a fall in titratable acidity from May to June, which they attributed to pasture feeding. It is probable that the decline in early winter is due to the high proportion of cows in advanced lactation, since this factor is known to lower the titratable acidity(35). In all probability too, the rise in early spring is due to the reduction of this disturbing factor and the introduction of more newly calved cows, the effect of

which would cause an increase in titratable acidity. The fall in May to June is therefore more likely to be due to grazing than to any lactation effect.

The true serum acidity (*s*) follows the titratable acidity curve in its essential details.

The overrun (*o*) tends to be high in spring and summer and lower in autumn and winter.

Caseinogen acidity shows a seasonal variation which is distinct from the other acidity factors, and, moreover, shows no close resemblance to the caseinogen curve (Fig. 3*b*). After a decline to January it rises sharply in February and then suffers a steady decline to July from which it quickly rises again to autumn. Its great similarity to the tricalcium phosphate and insoluble inorganic phosphate graphs (Figs. 6*a* and 8*a*) is significant.

Ratio *soluble inorganic* P_2O_5/*soluble* CaO (Fig. 11*b*). Since insoluble inorganic P_2O_5 is present almost entirely as tricalcium phosphate, which also accounts for the greater part of colloidal calcium, it was thought advisable to show the seasonal variation of the ratio of soluble P_2O_5 to soluble CaO. Considerable fluctuations are observed, but there appears to be a tendency for the spring and summer values to increase and those of autumn and winter to decrease.

Ratios to caseinogen

(*a*) *Acidity* (Fig. 12*a*). The effect of reducing the observed caseinogen acidity figures to the basis of 1 g. of caseinogen is very strikingly shown by comparing Figs. 12*a* and 11*a*. The peak of the graph is now transferred to May, and the subsequent decline continues until October.

(*b*) *Caseinogen* CaO (Fig. 12*b*). The most significant feature of this graph is the sudden fall from March to May and equally sudden recovery from May to July. That the minimum should coincide with the peak of the acidity graph is what might be expected, but in neither case can these effects be attributed entirely to the commencement of the grazing season, as the inflexions in the graphs are well established before this period.

(*c*) CaO *as* $Ca_3P_2O_8$. In view of the theory that colloidal calcium phosphate may be combined chemically to caseinogen, the ratio of CaO in this form to caseinogen is plotted in Fig. 12*c*. No constancy of this ratio is observed, in fact, the seasonal variation is very similar to that of the acidity per gram of caseinogen.

The variations in these ratios to caseinogen may be influenced by the lactation composition of the herd, but there is at present no means of verifying this possibility, since the effect of lactation on these factors is still obscure.

Reviewing the seasonal changes as a whole, it is evident that in some cases the lactation composition of the herd proves to be a disturbing factor. The problem is complicated by the influx of newly calved cows at the critical period at which pasture-feeding commences, and in some of the constituents under observation it is not possible to state the single effect of either condition. It has

therefore been the purpose of this section to present results as completely as possible, and to defer the drawing of definite conclusions until more is known of the lactation effects.

The low percentage of caseinogen in May, with its high acidity and correspondingly low calcium saturation, is of special interest. Fig. 13*a* indicates that in this month the ratio insoluble inorganic P_2O_5/colloidal CaO is at its maximum, a circumstance which might be expected to lead to the formation of dicalcium phosphate, leaving more calcium available to reduce the unsaturation of caseinogen. Yet under these circumstances, the greater part of the colloidal calcium is employed in the formation of basic calcium phosphate, leaving caseinogen in a very unsaturated condition. This condition is emphasized in Fig. 13*b*, showing the ratio of calcium as tricalcium phosphate to caseinogen calcium. While the mean value is 2·28, there is a sharp ascent to the maximum, 4·4 in May. These considerations will receive treatment in the following section.

Many investigators have studied the composition of milk from different points of view, and although published data sometimes show a lack of conformity, there is a general conviction that milk is in a state of equilibrium which is extremely sensitive to disturbing influences. For instance, Rice & Markley (36) conclude that an equilibrium exists between citric and phosphoric acids, caseinogen, and the bases. Sommer & Hart (37) found that the heat stability of milk was influenced by the ratio of phosphoric and citric acids to calcium and magnesium. This same ratio also influenced the alcohol test, where it was seen that the acids counterbalanced the effects of the two bases (38). The data presented in this paper were therefore examined with a view to finding what relationship exists between the various constituents under observation. The salient results are as follows.

Table V

Time of rennet coagulation min.	Serum acidity	% soluble inorganic P_2O_5	% soluble CaO	Ratio soluble inorganic P_2O_5/ soluble CaO	Approx. % soluble calcium citrate as CaO	% soluble organic P_2O_5
1·6–1·8	0·976	0·0893	0·0593	1·506	0·028	0·044
1·8–2·0	0·940	0·0890	0·0577	1·557	0·032	0·041
2·0–2·2	0·889	0·0852	0·0616	1·414	0·037	0·042
2·2–2·4	0·882	0·0841	0·0627	1·340	0·038	0·049
2·4–2·6	0·887	0·0859	0·0594	1·464	0·035	0·043
2·6–2·8	0·898	0·0840	0·0637	1·320	0·039	0·049
2·8–3·0	—	—	—	—	—	—
3·0–3·2	—	—	—	—	—	—
3·2–3·4	0·861	0·0812	0·0679	1·206	0·044	0·056

Rennet coagulation and serum acidity. These two values do not show the close relationship that might be expected, having regard to the fact that the serum acidity is so largely composed of acid phosphates.

Rennet coagulation and soluble inorganic P_2O_5. A somewhat closer relationship is seen to exist between these two values.

Rennet coagulation and soluble CaO. In the present series it is seen that

high soluble calcium is accompanied by a longer time of rennet coagulation, and vice versa. This result is surprising in view of the generally accepted fact that the calcium ion enhances the speed of rennet coagulation. Golding *et al.* (26), however, noticed a negative correlation between total calcium and the time of rennet coagulation.

Rennet coagulation and the ratio soluble inorganic P_2O_5/*soluble* CaO. The values for these two factors show a much more pronounced relationship; a result which suggests that the conditions known to influence the heat and alcohol coagulation of milk may also affect rennet coagulation. As citric acid was not determined, it is not possible to present the actual soluble citrate/calcium ratio. A very rough approximation to the percentage of soluble calcium citrate may be made by converting the serum acidity to $CaH_4P_2O_8$, and deducting the CaO in this form from the total soluble calcium. This approximation is based on two suppositions, (1) that acid calcium phosphate alone accounts for the serum acidity, and (2) that soluble calcium exists only as acid calcium phosphate and calcium citrate. Table V shows the relationship between rennet coagulation and the approximate estimate of calcium citrate obtained by this means. It would therefore appear that the presence of calcium as citrate nullifies the accelerating influence of calcium ions. As the total soluble citrate will be considerably more than this approximation, on account of its presence as alkali salts, it is likely that the total retarding effect on rennet action will be considerably more.

In order to check these deductions, it was decided to determine the effects of added calcium, phosphates, and citrates on the speed of rennet coagulation.

N/2 solutions of calcium lactate, calcium acetate, mono-, di- and trisodium citrates, disodium phosphate, and monopotassium phosphate were prepared and added to milk at the rate of 1 ml. per 100 ml.

The rennet tests were conducted as already described, and the effects of these additions are summarized as follows:

Acceleration	Retard
Calcium lactate	Trisodium citrate
Calcium acetate	Disodium citrate
Monosodium citrate	Disodium phosphate
Monopotassium phosphate	

Although this method of finding the conditions affecting rennet coagulation is open to the objection that changes in *p*H and ionic equilibria invariably occur when mineral salts are added to milk, yet the combined evidence supports the view that the phosphate citrate/calcium ratio influences rennet action as well as heat coagulation and the alcohol test. The retarding influence of tri- and dicitrates, and diphosphates explains why the anticipated relationship between rennet action and soluble calcium, serum acidity or soluble inorganic phosphate is not evident in this series of tests.

Rennet action and soluble organic P_2O_5. It is interesting to note that low percentages of soluble organic phosphorus are associated with shorter times of

rennet coagulation, and vice versa, although it is impossible to state whether this is a primary or secondary effect.

Overrun and serum acidity (Table VI). Since the true acidity of the serum is largely due to monocalcium phosphate, one would expect to find it closely related to the overrun. From the equations shown below, the serum acidity/overrun ratio should approximate to 3/1. The presence of acid phosphates other than calcium would tend to increase this value. The mean ratio found in

Table VI

Serum acidity	0·76–0·80	0·80–0·84	0·84–0·88	0·88–0·92	0·92–0·96	0·96–1·00	1·00–1·04	1·04–1·08
Overrun	0·347	0·328	0·371	0·365	0·394	0·400	0·369	0·383

this series of analyses is 2·46. From this, the conclusion may be drawn that dicalcium phosphate other than that formed by direct neutralization participates in equation B. In all probability, the sodium phosphate formed in equation A combines with some of the calcium citrate to furnish more dicalcium phosphate:

$$CaH_4P_2O_8 + 2NaOH = CaHPO_4 + Na_2HPO_4 + 2H_2O, \quad \text{.....(A)}$$

$$3CaHPO_4 = Ca_3P_2O_8 + H_3PO_4. \quad \text{.....(B)}$$

It is no doubt due to this fact that the relationship between the values of overrun and serum acidity given in Table VI is not so close as might have been expected.

The condition of caseinogen in milk, and its association with other elements and compounds, particularly calcium phosphate, has been the subject of much study and experiment, but there is no unanimity of opinion regarding the precise form of this association. Thus Pyne (1), Kometiani (39), Lindet (40), Lehman (11), Hammarsten (41), and György (42) consider that colloidal calcium phosphate is chemically united to caseinogen, while Soldner (12), Van Slyke & Bosworth (43), Porcher (44) and Powell & Palmer (7) either reject the theory of association entirely or concede only a protective role to the protein. The relationship existing between caseinogen and other values found in this work was therefore examined in the hope that some further evidence would be forthcoming to assist in the elucidation of this problem.

Caseinogen and tricalcium phosphate. Fig. 14 shows that these two values move in the same direction, but that the linear relationship is not upheld at lower values of caseinogen. It was observed earlier in this paper that the ratio of calcium as tricalcium phosphate to caseinogen was not constant.

Caseinogen acidity per gram caseinogen and serum acidity (Table VII) indicates that an equilibrium exists between these two acidity factors.

Caseinogen acidity and caseinogen CaO *per gram caseinogen respectively.* Although a high degree of calcium saturation is reflected in a correspondingly lower acidity and vice versa, Table VII indicates a considerable scattering of values at low acidities.

Caseinogen acidity and CaO *as tricalcium phosphate, per gram caseinogen respectively.* Fig. 15 shows that a very close linear relationship exists between these factors, a result which is significant, since it throws light on the association of these two colloids. It appears that while colloidal tricalcium phosphate is certainly associated with caseinogen, the determining factor is not so much the actual amount of the protein present in milk, but its degree of acidity.

Table VII

Caseinogen acidity per gram caseinogen	Serum acidity	Caseinogen CaO per gram caseinogen
0·28–0·29	0·874	0·0156
0·29–0·30	0·850	0·0140
0·30–0·31	0·897	0·0139
0·31–0·32	0·880	0·0138
0·32–0·33	0·960	0·0147
0·33–0·34	0·903	0·0141
0·34–0·35	0·913	0·0148
0·35–0·36	1·066	0·0116
0·36–0·37	0·980	0·0112
0·43–0·44	—	0·0063

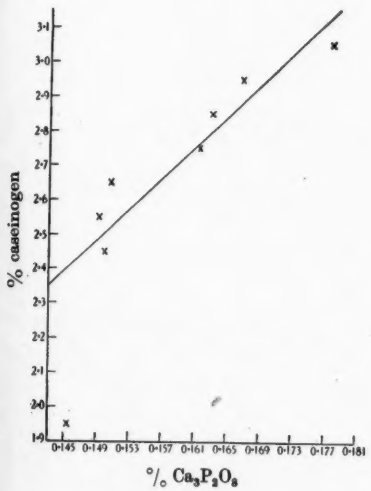


Fig. 14.

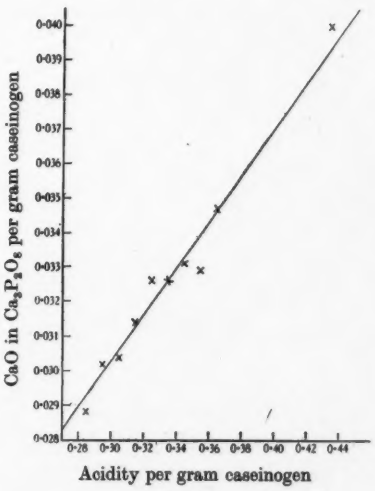


Fig. 15.

Further, the basicity of the tricalcium phosphate is practically equivalent to the acidity of the caseinogen. The mean percentage CaO as $Ca_3P_2O_8$, 0·0843, is equivalent to 1·00 ml. *N* acid, while the mean acidity of caseinogen is 0·86 ml. *N*. These results favour the existence of a physical association between the two colloids rather than direct chemical union; for it is difficult to conceive an acid caseinogen combining with a basic colloid in such a way as to leave the acidity unreduced. If they were combined under this extraordinary condition, then a high degree of acidity would be associated with a low percentage of tricalcium phosphate and vice versa, whereas the results of the present work show the

reverse relationship to apply. Further, if such combination were to occur, it would presumably proceed according to the following equation:

$$[\text{Protein}=(COOH)_2+Ca_3P_2O_8]=[\text{Protein}=(COO)_2Ca+2CaHPO_4].$$
Complex A Complex B

But complex B would not give an acid reaction, neither would it develop alkalinity with potassium oxalate, and the increase in protein CaO would bring the latter to 2·45 per cent CaO, an amount in excess of that generally accepted.

In a previous paper(2) it was found that when lactic acid was allowed to develop in milk the tricalcium phosphate was dissolved with greater ease than caseinogen calcium, and that the increase in acidity of the protein was related to its depletion of calcium rather than losses of tricalcium phosphate. This evidence was therefore not compatible with the theory of chemical union between the two colloids. Powell & Palmer(7) studied the effect of heat on the rennet coagulation of calcium caseinogenate and calcium-caseinogen-phosphate complexes and could find no evidence to support the view that in milk chemical union with the phosphate took place.

The evidence in favour of physical association is therefore accumulating, and the present work indicates that the basic phosphate held by caseinogen is in some manner related to the acidity of this protein. A state of equilibrium is found to exist between the basic phosphate and caseinogen acidity on the one side, and serum acidity on the other.

Caseinogen	$Ca_3P_2O_8$	Serum acidity
Acidity 0·86 ml. *N*	Basicity 1·00 ml. *N*	0·91 ml. *N*

Summary

1. The means and standard deviations of analytical data obtained from fifty-four samples of mixed milk (alternate a.m. and p.m.) and whey from the College herd of Dairy Shorthorns are presented in Table I. The samples were taken at intervals during the period late September 1935 to November 1936.

2. While no outstanding differences in composition are evident between morning and evening samples, the time of rennet coagulation was appreciably greater with the evening's samples. Evidence is presented to show that the longer time of cold storage of these samples must be regarded as a contributory cause of this effect.

3. In the discussion of the mean values, special treatment has been given to the partition of calcium and phosphorus between the four phases, soluble and insoluble, organic and inorganic. The caseinogen values are examined in the light of current theories.

4. Monthly averages are plotted in Figs. 2–13, together with temperature and lactation details of the herd. Pronounced disturbances occur in most constituents during the course of the year, the most general periods being January to February, April to May, and late summer to early autumn.

5. It is difficult to determine whether the May variations are due to the commencement of grazing or to the higher proportion of cows in early lactation, since both conditions coincide.

6. Although the time of rennet coagulation decreased with increasing serum acidity and with an increasing soluble inorganic P_2O_5/soluble CaO ratio, it was found to increase with increasing soluble CaO. Since di- and tricitrates and diphosphates were found to prolong rennet coagulation, it was concluded that these disturbing factors were at least partly responsible for the anomalous effect of soluble calcium.

7. The relationship between caseinogen and other constituents is examined, and the evidence of the present work supports the view that this protein is associated with tricalcium phosphate in a physical rather than chemical manner. The amount so held by caseinogen is closely related to the acidity per gram of caseinogen.

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157. THE CREAMING POWER OF HEATED MILK. THE RELATIONSHIP BETWEEN THE DENATURATION OF ALBUMIN AND GLOBULIN AND THE REDUCTION IN CREAMING POWER

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(With 2 Figures)

The heating of milk at temperatures of 63° C. and above reduces its creaming power, and results in a slower rate of cream formation and a smaller final cream volume. Hence the effect of pasteurization on creaming is of commercial significance, as the purchaser of milk usually estimates its quality by a glance at the depth of the cream layer. Whittaker *et al.* (1) found that pasteurization for 30 min. at 63° C. reduced the cream volume by about 8 per cent, but that 30 min. at 61·7° C. resulted in an unchanged, or in some cases slightly increased, cream volume. Dahlberg & Marquardt (2) found that heating for 30 min. at temperatures of 60–63° C. did not greatly affect the cream volume, but that at higher temperatures (3) there was a marked reduction. Orla-Jensen (4) showed that heating for 30 min. at 45–62° C. increased the depth of the cream layer. The maximum increase was at 61° C.; as the temperature was increased above 62° C. the depth of the cream layer decreased rapidly. Clearly the changes taking place at 60–63° C. need special consideration.

The reduced creaming power of heated milk was believed to be due to the loading of the fat globules with coagulated lactalbumin, until Rahn (5) showed that it could be restored to a normal value by the addition, after the heating, of such colloids as gelatin, gum tragacanth and albumin. He also showed that these substances increased the creaming power of unheated milk by facilitating the aggregation of the fat globules into clumps of greater buoyancy. Weinlig (6) followed this by showing that the heating of milk reduced its creaming ability as it destroyed the clumping of the fat globules, and Rahn (7, 8) confirmed that hydrophilic colloids restore the creaming by restoring the ability of the fat globules to form clusters. In agreement with the above observations, van Dam & Sirks (9), Palmer *et al.* (10), and Orla-Jensen (11) demonstrated that the whey proteins, lactalbumin and lactoglobulin, are effective promoters of fat-globule clustering and rapid creaming provided that their solutions have not been heated, whereas calcium caseinate hinders creaming. Palmer *et al.* reached the general conclusion "that both exhaustiveness of rise of fat and greater volume of cream are promoted by the more truly hydrophilic colloids and depressed by those with hydrophobic properties".

The results which have been outlined above support the suggestion first made by Weinlig(6) that in unheated milk the soluble proteins cause the clumping of the fat globules by forming on them an adsorbed colloidal envelope which admits of their adhesion as they collide during their movement; and that in heated milk the clumping of the globules is reduced because the concentration of the soluble proteins is reduced by denaturation. The addition of hydrophilic colloids to the heated milk restores the adsorbed layer. This suggestion that the denaturation of albumin and globulin is the cause of the diminished creaming of heated milk has not found general approval. Palmer *et al.*(10) considered that the heating of milk reduced creaming by increasing the hindering action of calcium caseinate, and stated that experiments on the heat coagulation of albumin and globulin "indicate clearly that appreciable coagulation of albumin and globulin does not occur under the influence of such time and temperature factors as seriously affect creaming".

A proper understanding of the changes underlying the reduced creaming power of heated milk has been handicapped by the lack of reliable data on the extent to which albumin and globulin are denatured when milk is heated. Such data have recently been provided(12, 13), showing that there is appreciable denaturation on heating milk for 30 min. at as low a temperature as 63° C. An investigation of the effect of heat on the creaming power and on the denaturation of the soluble proteins of the same milk is clearly required. A search of the literature has not revealed any such investigation. The denaturation of albumin and globulin involves a change in the colloidal nature of the proteins from the typically hydrophilic to the hydrophobic type. It is therefore reasonable to assume that the reduction in hydrophilic colloids and increase in hydrophobic colloids when milk is heated are responsible, if only partly, for the reduced creaming power of the heated milk. To obtain information concerning the relationship between the denaturation of albumin and globulin and the reduction in creaming power of heated milk, a series of determinations has been made of the amounts of albumin and globulin denatured, and of the changes in creaming power produced, by heating samples of milk for 30 min. at various temperatures.

Experimental

Heating of milk samples and measurement of creaming power

The milk samples were taken in narrow-necked 200 ml. flasks and immersed in thermostats containing water covered with a layer of oil. During the rise of temperature the milk was stirred. The flasks were then stoppered and shaken at intervals. The flasks were filled with milk to such a level that, on heating, the milk expanded just into the neck. This procedure reduced considerably the exposed surface; there was no skin formation, and evaporation from the milk was negligible.

After the required periods the flasks were removed from the thermostats and cooled to 12° C. The milk samples were set to cream in 100 ml. measuring

cylinders at room temperature of 15° C. The height of the column of milk was 15 cm. The depth of the cream layers was measured with dividers.

Determination of the extent of the denaturation of albumin and globulin

Casein was precipitated from unheated milk, and casein plus denatured albumin and globulin from the heated samples, at *p*H 4·7, by the addition of an acetic acid-sodium acetate mixture. The total proteins were precipitated with trichloracetic acid. Details of the semi-micro-Kjeldahl method employed for the determination of the N distribution have been given previously (13).

Preliminary experiments

Preliminary experiments were made in which only the changes in creaming power were measured, in order to ascertain which temperatures below 60° C. merited investigation and to provide data for the selection of a suitable creaming time for the final experiment. A typical set of results is given in Table I, which shows that the maximum increase in creaming power followed heating for 30 min. at 60° C. Heating for the same time at 57·5° C. and below resulted in a slightly smaller increase, and at 63° C. in a definite decrease in creaming power as compared with raw milk.

The final experiment

Samples from a bulk of fresh milk were heated for 30 min. at 57·5, 60·0, 61·5, 63·0, 65·0, 67·5, 70·0, 74·8, and 80·0° C. The depth of the cream layers was measured after 4 hours.

RESULTS

The protein distribution of the milk sample treated in the final experiment is shown in Table II, and the results of the heating on the rate of creaming and on the extent of the denaturation of albumin and globulin in Table III. The effect of the temperature of heating on the rate of creaming is recorded in Fig. 1.

Table I. *Effect of heating on the creaming power of milk*

	Time of heating, 30 min.			
	Depth of cream layer in mm.			
Temp. of heating	Creaming time in hours			
° C.	2	4	8	24
Raw milk	3·5	7·0	9·0	10·0
63·0	3·0	6·3	8·5	9·5
60·0	11·0	12·0	12·0	13·0
57·5	9·3	11·0	11·5	12·3
55·5	9·0	10·5	11·5	12·3
50·0	8·7	10·3	11·5	12·3

Table II. *Composition of the raw milk*

N fraction	mg. %
Total N	516·0
Casein N	400·8
Albumin and globulin N	67·3
Proteose N	21·7
Non-protein N	26·2

Table III. *Effect of heating on the creaming power and on the solubility of the albumin and globulin of milk*

Time of heating, 30 min.; creaming time, 4 hours

Temp. of heating ° C.	Depth of cream layer mm.	Percentage of total albumin and globulin denatured
Raw milk	12·3	—
57·5	14·0	2·7
60·0	15·3	5·5
61·5	13·3	7·4
63·0	10·5	11·0
65·0	5·3	15·6
67·5	2·5	20·5
70·0	2·0	33·4
74·8	2·0	82·0
80·0	2·3	99·1

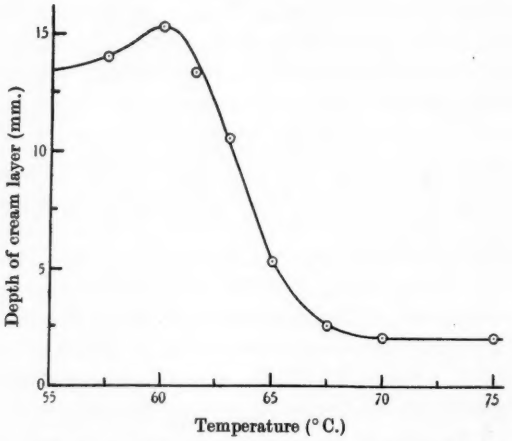

Fig. 1. Effect of heating on the creaming power of milk.

Discussion of results

The effect of temperature of heating on creaming power

From Fig. 1 it will be seen that after heating for 30 min. at 62° C. the creaming power of the heated milk equals that of the unheated milk. Heating at temperatures below 62° C. increases the creaming power, the maximum increase being at 60° C. (24 per cent), and above 62° C. progressively decreases it (15 and 57 per cent at 63 and 65° C. respectively). In stating the actual magnitude of changes in creaming power the time of creaming is important, as shown in Table I. The milk samples exhibiting increased creaming power not only gave a greater final cream volume but also formed their cream layers more quickly than unheated milk.

The relationship between protein denaturation and creaming power

The data of Table III and the curve of Fig. 1 indicate that at least two major factors are concerned in the effect of heating on creaming power, and that the temperature of 60° C. is of importance. Clearly, at lower temperatures heating facilitates creaming through the operation of some factor not investigated in the experiment. At higher temperatures a second factor becomes dominant, and with increase of temperature it rapidly reduces the creaming power. This second will be considered to be the denaturation of soluble protein. Some slight denaturation of protein was found at 57·5° C. and more at 60° C., hence the increase of creaming power between these temperatures must be due to the influence of the first factor predominating over the influence of the second.

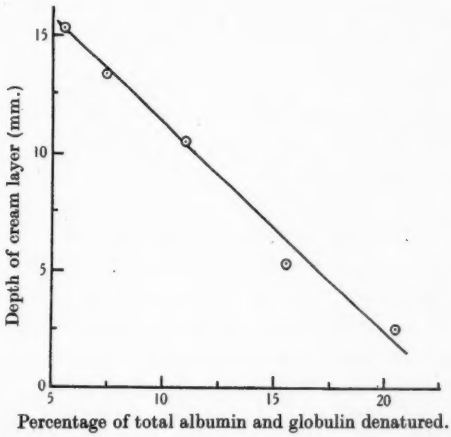

Fig. 2. Relationship between extent of protein denaturation and creaming power.

The relationship between the extent of the denaturation of albumin and globulin and the creaming power of the milk after heating at 60–67·5° C. is shown in Fig. 2 by plotting the percentage of the total albumin and globulin denatured against the depth of the cream layer. (The results of heating at 70° C. and above are not included, as the depth of the cream layer at those temperatures was extremely small, did not change with temperature, and undoubtedly represented a minimum creaming capacity due to the ascent of a few single fat globules. The results at 70° C. and above are not related to the hydrophilic protein content of the milk, or based on fat-globule clumping.) It will be seen that the points fall very near to a straight line, indicating that on raising the temperature from 60 to 67·5° C. the marked reduction in creaming power is proportional to the percentage of the total albumin and globulin denatured, and therefore proportional to the residual albumin and globulin content of the milk.

The creaming of milk is dependent on the ability of the fat globules to form large clumps, the clumping being promoted by hydrophilic colloids adsorbed at the fat globule-skim milk interface. The amount of albumin and globulin in raw milk is below the optimum for the promotion of maximum clumping, as the addition of hydrophilic colloids increases creaming in spite of an increase in viscosity. When the concentration of hydrophilic colloids at the fat globule-skim milk interface is reduced in heated milk by the denaturation of albumin and globulin, the extent of clumping and creaming is also reduced. The clumping and creaming of heated milk may be restored by increasing the hydrophilic colloid content, and one is justified in considering the denaturation of albumin and globulin to be a major factor in the reduced creaming of heated milk.

The increase in creaming power of milk heated at temperatures below 62° C.

It has been shown that heating milk for 30 min. at temperatures up to 62° C. increases the creaming power above that of the raw milk, and an explanation may be suggested on the basis of viscosity changes, for Taylor (14) and Weinlig (6) found that whole-milk samples that had been heated for 30 min. at various temperatures and then cooled had gradually decreasing viscosities as the temperature increased to 60° C. Above 65° C. the viscosity began to increase again and reached the raw milk value at about 70° C. At temperatures up to 60° C.—at which the maximum creaming power was observed—the decrease in viscosity on heating assisted the ascent of fat globules and clumps of globules to a greater extent than the slight reduction in soluble proteins (e.g. 2·7 per cent at 57·5° C.) retarded the clumping of fat globules. But above 60° C. there is no further reduction in viscosity to assist creaming, and the progressive denaturation of albumin and globulin has its effect on the clumping power of the fat globules. Troy & Sharp (15) demonstrated that the ascent of clumps of fat globules follows Stokes' Law, so that at 62° C. when heated milk has the same creaming power as raw milk, the reduction in viscosity exactly compensates for the smaller average size of ascending clumps.

It is thus possible to explain the relationship between the temperature of heating and the creaming power of milk, in fact the shape of the curve of Fig. 1, by considerations of viscosity changes followed by denaturation of albumin and globulin. When the work which has been described was carried out, it seemed unnecessary to discriminate between the albumin and globulin and to make separate determinations of the extent of their denaturation, as the literature contained evidence that a large variety of hydrophilic colloids, including the mixed whey proteins, could promote the creaming of milk. But there is the possibility that the denaturation of the globulin alone is largely responsible for the reduction in creaming power. Hekma & Sirks (16) and Brouwer (17) found blood-serum globulins, especially of the euglobulin type, to be particularly effective promoters of cream rising. The writer has since made some preliminary experiments in which the extent of globulin denaturation has been related to

the reduction in creaming power of some heated milk samples. There are difficulties in the determination of small changes in the initially extremely small globulin content of milk, and the results obtained to date need confirmation. They suggest that the reduction in the creaming power is proportional to the extent of the denaturation of globulin, and that the globulin of milk is completely denatured by heating the milk for 30 min. at a temperature of 70° C. The last point is most significant. It means that the reduction of the creaming power of milk to a negligible value that takes place as the temperature of heating is raised from 60 to 70° C. is to be correlated with the denaturation of all of the globulin.

SUMMARY

1. The effect of heating on the creaming power of milk and the rôle of truly hydrophilic colloids in increasing the creaming power of raw and of heated milk are discussed. The suggestion is made that a proper understanding of changes underlying the reduced creaming power of heated milk has been handicapped by a lack of reliable data on the extent to which the hydrophilic colloid content of milk is reduced by denaturation of albumin and globulin.

2. Information is obtained concerning the relationship between the denaturation of albumin and globulin and the reduction in creaming power from a series of determinations of the amounts of protein denatured, and of the changes in creaming power produced, by heating samples of the same milk for 30 min. at temperatures of 57·5, 60·0, 61·5, 63·0, 65·0, 67·5, 70·0, 74·8 and 80·0° C.

3. After heating for 30 min. at 62° C. the creaming power of the heated milk equals that of raw milk. Heating below 62° C. increases the creaming power, the maximum increase being at 60° C., and heating above 62° C. progressively and rapidly reduces it.

4. The reduction in creaming power which follows the increase of temperature from 60° C. upwards is proportional to the percentage of the total albumin and globulin denatured, and therefore proportional to the residual soluble albumin and globulin content of the milk. The increased creaming power, as compared with raw milk, of samples heated at temperatures up to 62° C. is explained on the basis of viscosity changes.

5. The conclusion is reached that the denaturation of albumin and globulin, and probably especially of globulin, is a major factor in the reduction in the creaming power of heated milk. The relationship between the temperature of heating and the creaming power of heated milk is explained by considerations of viscosity changes and soluble protein denaturation.

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158. THE NUTRITIVE VALUE OF RAW AND PASTEURIZED MILK FOR MICE

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From the point of view of both human and veterinary medicine it is clearly of importance to determine whether pasteurization brings about any significant diminution in the nutritive value of cow's milk. Since the food requirements of man and of different animals are not identical, the answer to this question will necessarily depend upon the particular animal chosen for experimental study. Wilson *et al.* (1) have recorded the results of a comparative experiment on calves, in which alternate animals born into a herd were fed on raw and holder-pasteurized milk. Judged by increase in weight during an 8-week period the calves fed on pasteurized milk behaved as well as those on raw milk, and no evidence was obtained to suggest that the nutritive value of the milk had been in any way diminished by the heat treatment it had received.

In the present paper the results of an experiment on mice are recorded. These animals were selected, partly because they could be used in large numbers, and partly because, in continuation of previous immunological experiments on mice, we were anxious to determine whether small dietetic changes were likely to bring about an alteration in their resistance to infection. Little or no work appears to have been carried out on the effect of raw and pasteurized milk on mice. A considerable number of papers, however, have been published on rats. Many of these have already been summarized by Stirling & Blackwood (2) or by Kon (3, 4). In addition, we may refer to papers by Mattick & Golding (5), Channon & Channon (6), and Kon and his colleagues (7).

The findings of these various workers have been somewhat conflicting. On the whole, however, they may be summarized by saying that cow's milk is an unsatisfactory diet for rats. It is deficient in iron, copper, and manganese, and is unable to prevent the development of anaemia in animals fed on it exclusively. It likewise appears to be deficient in substances that are required for satisfactory reproduction and for rearing of the young. How far this is due to deficiency of vitamin B is still obscure. With regard to the effect of pasteurization, no satisfactory evidence has yet been brought forward to show that, pint for pint, holder-pasteurized milk is inferior in nutritive value to raw milk. It must, of course, be noted that vitamin C is not required by the rat, and that the partial destruction of the vitamin B complex recorded by Kon (7) is detectable only at a low level of milk intake (8 ml. daily).

Preliminary experiments, Nos. 1–25

In the preliminary experiments, parti-coloured mice were used. They were generally 10–14 g. in weight; they had been brought up by does on a diet of oats and dilute boiled milk; and they were free from any demonstrable bacterial infection. They were kept in groups of five, either in zinc cages of the pill-box type (Topley (8)) or in open wire-mesh cages. Bucks and does were run separately, except during breeding times, when one buck was allotted to four or five does. Weights were taken once a week. The number of litters born was registered, but the number of mice in each litter at birth was not counted, since many does ate their young within the first 24 hours of parturition. For recording purposes a litter was not considered as having been reared until the mice were 28 days old and were ready for weaning. Some experiments were terminated after opportunity had been afforded for each doe to have one litter, but in other experiments an attempt was made to obtain two or three litters in succession from each doe.

The basal diet consisted either of raw mixed milk, or of the same batch commercially pasteurized by the holder method at 145° F. (62·8° C.) for 30 min., together with a biscuit prepared from white flour and water. In some experiments this diet was supplemented by a mineral solution consisting either of a mixture of copper sulphate and ferric ammonium citrate, or of the same mixture plus manganous chloride. The mineral mixture was generally added to the milk in such a proportion that 1 c.c. of the treated milk contained 0·01 mg. of copper sulphate, 0·005 mg. of ferric ammonium citrate, and 0·001 mg. of manganous chloride. In other experiments a supplement of Yeastrel was given, this substance being a rich source of vitamin B, while in still other experiments the basal milk-and-biscuit diet was supplemented by both mineral mixture and Yeastrel.

On all diets the milk was given to the mice in inverted tubes twice daily, the amount supplied being in excess of consumption. Biscuit was distributed once a day *ad lib.* Needless to say, all animals in the "raw" and "pasteurized" groups were treated alike except in regard to the nature of the milk they were receiving.

Results of preliminary experiments

Some of the results of the early experiments were communicated to the Society of Chemical Industry as part of a special symposium on Bread and Milk and were published (Wilson & Cowell (9)) in the Society's *Journal*. There is therefore no need here to do more than summarize the main conclusions reached.

(1) It was found that both raw and pasteurized milk, in conjunction with a white-flour biscuit, were quite inadequate to promote normal growth. Young mice on this dietary generally died within a few weeks in an emaciated and often severely anaemic condition.

(2) The addition of a copper and iron mixture to the basal diet improved growth and development and prevented the occurrence of anaemia, but did not usually suffice to enable the does to rear their litters.

(3) The addition of Yeastrel to the basal diet overcame this difficulty, and when both mineral mixture and Yeastrel were added, growth, reproduction, and rearing of the litters appeared to be almost as satisfactory as on a stock diet of oats and dilute boiled milk. It may be noted in parenthesis that such a stock diet has since been found by Watson (10) to be a comparatively poor diet for the development of mice.

(4) With half-grown animals brought up on stock diet and then transferred to the experimental diet, no significant difference was found between those fed on raw and those fed on pasteurized milk in relation to general development, survival time, number of litters produced, or the proportion of young reared.

Table I. *Preliminary experiments. Synoptic table comprising the results of six experiments in which the average weight in grams of mice was compared at the beginning and end of a 12-week period on a diet of raw or pasteurized milk and biscuit, supplemented by mineral mixture, or Yeastrel, or both. Milk given in inverted tubes*

	Raw milk				Pasteurized milk				Raw and pasteurized milk		
	No.	Initial wt.	Final wt.	S.D. final wt.	No.	Initial wt.	Final wt.	S.D. final wt.	O.D.	S.E. diff.	O.D. / S.E. diff.
Bucks	29	12·93	25·66	4·55	25	13·20	23·28	4·84	+2·38	1·28	+1·86
Does	43	12·56	23·72	4·39	39	12·36	24·62	2·12	−0·90	0·75	−1·47
Bucks and does	72	12·71	24·50	4·56	64	12·69	24·09	3·53	+0·43	0·70	+0·61

S.D. = standard deviation. O.D. = observed difference between the final weight of the raw and pasteurized mice, making allowance for the difference in their initial weight. S.E. diff. = standard error of this difference. + means in favour of raw milk. − means in favour of pasteurized milk.

Note. It would have been more satisfactory to have worked out the standard deviations of the increases in weight rather than of the final weights, so as to avoid making an allowance for the difference in the initial weights. The results, however, would probably have been much the same.

(5) On the other hand, with mice brought up from birth by does on a milk-and-biscuit diet, supplemented by Yeastrel and a mineral mixture, the average weight of the animals at weaning time was significantly higher in the raw than in the pasteurized groups.

The evidence on which these conclusions were based was given in tabular form in the paper already referred to. We may, however, add the figures on weight increase, which were not ready at the time of the symposium (Table I). These figures refer exclusively to mice that were observed for a period of 12 weeks after weaning. It will be seen that among the bucks the animals on raw milk gained rather more than those on pasteurized, while among the does the reverse occurred. In neither instance was the difference significant. Taking all animals together there was a slight but quite insignificant advantage in favour of the mice belonging to the raw-milk groups.

One further observation may be recorded. It was found that zinc cages of the pill-box type were unsatisfactory for breeding purposes. The number of litters produced was small and the proportion reared was very low. On the other hand, open wire-mesh cages appeared to be much more satisfactory. Investigations carried out on this question by Watson(10) have since shown that zinc, which tends to flake off cages of the pill-box type, is toxic to mice when ingested with the food, and interferes particularly with their reproductive and rearing ability.

Intermediate experiments, Nos. 26–38

As a result of the preliminary experiments it was realized that if a satisfactory comparison was to be made between animals fed on raw and pasteurized milk, it would be necessary (1) to supplement the diet with a mineral mixture, (2) to give in addition Yeastrel to the breeding mice, and (3) to carry out all experiments either in open wire-mesh or in glass cages. In the intermediate group of experiments about to be described these conditions were observed.

Besides satisfying these requirements we should have liked to standardize the volume of milk ingested by the two groups of animals. This, however, presented considerable difficulties. The consumption of milk was so variable, not only from cage to cage, but from animal to animal, that any attempt to standardize its amount would have placed us in a dilemma. The intake of each mouse would have had to be fixed either (1) at the smallest amount consumed by any one mouse, in which case many of the animals would have suffered from relative starvation, or (2) at the largest amount consumed by any one mouse, in which case gross differences in the volume taken by individual animals would have resulted. In practice we adopted an intermediate position, regulating the volume of milk more or less in accordance with the requirements of the average mouse. It was realized, of course, that if one group of animals drank more than the other, the result would not afford a strict comparison, volume for volume, between the nutritive value of raw and pasteurized milk. Nevertheless it seemed reasonable to believe that, if there was any substantial difference between these two types of milk, it should be revealed by the method we proposed to adopt.

All mice were weaned at 4 weeks. If they had been brought up by does on the stock diet of oats and dilute boiled milk, each litter was divided into two groups labelled raw and pasteurized, having as nearly as possible the same sex distribution and weight. If they had been brought up by does on a diet of raw or pasteurized milk plus biscuit, mineral mixture, and Yeastrel, every litter, independent of sex distribution and weight, was used to make up the corresponding raw or pasteurized group. As soon as all the mice had been assembled—a process that usually took 1–2 weeks—they were distributed into open wire-mesh cages, having a wire-mesh bottom through which the excreta fell into a tray containing sawdust. The dietary consisted, as in the preliminary experiments, of raw or pasteurized milk and white-flour biscuit. To the milk,

which was given in inverted tubes, was added a mineral mixture. In Exp. 26, and during part of Exps. 27–30, the mineral mixture was of the same composition as that given on p. 204. Later it was made rather stronger, and added in such a proportion that 1 c.c. of milk contained 0·109 mg. of ferric ammonium citrate, 0·0785 mg. of copper sulphate, and 0·072 mg. of manganous chloride. A mouse consuming 5 c.c. of milk daily should therefore have received approximately 0·1 mg. of iron, 0·01 mg. of copper, and 0·01 mg. of manganese over and above the small quantities of these substances already present in the milk. The mice were weighed weekly, and observed for a time that varied in different experiments from 7 to 19 weeks. All animals dying were submitted to a bacteriological post-mortem examination.

The does were then separated for breeding. About five does were run in an open wire-mesh cage with one buck. They were given raw or pasteurized milk containing mineral mixture plus 3 per cent Yeastrel, together with the ordinary white-flour biscuit. As soon as a doe showed signs of pregnancy, it was transferred to a glass cage of the pill-box type having a finely perforated zinc lid. After parturition the young were kept with the mother for 4 weeks. They were then removed and placed on a diet free from Yeastrel.

Mice in Exps. 26–33 were derived from the litters of does fed on the stock diet of oats and boiled milk diluted with an equal volume of water. Mice in Exps. 34–38 were derived from the litters of does fed on raw or pasteurized milk, supplemented by mineral mixture, Yeastrel, and biscuit; they constituted in fact the second generation fed on a raw or pasteurized milk dietary.

Apart from the pasteurized milk groups in Exps. 35 and 36, which were given a special supplement of 1·5 per cent of orange juice, all mice were treated alike in every respect except in relation to the milk, which was either raw or pasteurized.

Results of intermediate experiments

In considering the results of these experiments it will be convenient to deal with the rate of growth and the breeding performance separately.

Rate of growth.

The growth of the mice as revealed by the average initial and final weights is recorded in Table II. It will be noted that the initial weight of the mice in the raw and pasteurized groups was not identical. The reason for this is twofold. (1) In Exps. 26–33 mice were taken from litters brought up on a diet of oats and dilute boiled milk and divided into two groups of approximately the same weight. Since, however, not all litters were weaned simultaneously, it was necessary to add young mice for a period of a week or two to the raw and pasteurized groups in order to bring the numbers up to a satisfactory level. During this time the weight of the original mice in the two groups often increased at a different rate, so that the average weight of the mice when all of the animals had been collected was no longer the same. (2) In Exps. 34–38 the mice were taken from litters brought up on diets containing raw or

Table II. *Intermediate experiments. Growth of mice on a diet of raw or pasteurized milk supplemented by a mineral mixture and a white-flour biscuit. Milk given in inverted tubes.* (*Weights recorded in grams*)

Exp. no.	Length of exp. in weeks	Group	Bucks No. Start	Bucks No. End	Bucks Average initial wt.	Bucks Average final wt.	Bucks S.D. final wt.	Does No. Start	Does No. End	Does Average initial wt.	Does Average final wt.	Does S.D. final wt.	Remarks
26	7	Raw	35	34	9·74	21·91	4·04	29	26	9·72	21·23	4·00	Litters from does fed on oats and dilute boiled milk
		Past.	35	30	9·71	18·53	6·42	29	23	9·83	16·83	5·98	
27	12	Raw	15	14	14·80	24·93		8	8	12·13	19·50		,,
		Past.	15	5	14·07	28·00	R. 4·47	8	8	13·75	20·62	R. 4·55	
28	10	Raw	31	30	14·87	24·00	P. 6·78	25	25	16·99	21·80	P. 5·07	,,
		Past.	29	26	15·21	23·03		26	24	16·35	21·71		
29	10	Raw	33	32	17·06	25·19	4·32	29	29	16·41	20·52	3·13	,,
		Past.	32	30	17·65	25·60	6·27	29	29	16·06	19·86	2·98	
30	13	Raw	14	14	15·14	25·93	3·27	12	12	14·83	24·92	3·88	,,
		Past.	14	14	15·00	22·36	4·19	12	12	13·72	22·58	4·07	
32	14	Raw	32	24	13·68	25·58	3·67	31	30	13·87	21·43	4·94	,,
		Past.	31	25	13·45	22·76	6·60	31	27	14·09	19·56	4·41	
33	16	Raw	10	10	18·30	24·70	4·10	13	11	13·77	17·55	4·10	,,
		Past.	10	9	16·90	21·44	5·50	13	12	13·92	14·08	5·00	
34	13	Raw	18	16	14·39	25·88	3·72	33	26	15·21	18·88	3·73	Litters from does in preliminary experiments fed on raw or pasteurized milk
		Past.	33	25	13·42	18·24	5·55	23	20	14·52	17·60	5·85	
35	18	Raw	25	21	12·32	25·81	3·45	31	24	11·37	18·17	3·55	Litters from does fed on raw or pasteurized milk in Exp. 27
		Past.	26	14	11·73	25·21	4·12	37	22	11·02	17·50	3·70	
36	16	Raw	35	22	15·74	22·91	5·42	33	18	15·15	21·22	4·27	Ditto in Exp. 28
		Past.	28	14	13·71	22·64	5·57	20	13	13·35	19·85	6·66	
37	19	Raw	15	6	16·33	25·00	4·08	12	9	15·00	18·67	3·90	Ditto in Exp. 29
		Past.	20	13	13·95	23·23	3·99	17	16	13·29	20·06	3·01	
38	14	Raw	33	26	15·00	19·27	4·63	42	32	12·73	15·78	3·57	Ditto in Exps. 30 and 32
		Past.	26	21	13·07	20·33	5·29	34	23	12·02	16·96	4·61	

S.D. = Standard deviation. *Note.* No Exp. 31 was made.

pasteurized milk, and added to the corresponding experimental groups without any selection on the basis of sex or weight. Since the mice brought up on raw milk were usually heavier than those brought up on pasteurized milk, the difference in the average initial weight of the mice in the two groups of the growth experiment was sometimes considerable.

It was for these reasons that we decided to pay attention in the intermediate and subsequent groups of experiments to the *final weight* of the mice rather than to their increase in weight. To take only their increase in weight would have meant neglecting the various factors that were responsible for the higher average weight of the raw mice at the beginning of so many of the experiments, and so to weight the odds slightly against the raw-milk group. If the increase in weight had been taken, the significance of the difference between the raw and pasteurized groups would have been diminished in most of the experiments. Table III summarizes the results given in Table II. It will be seen that the final weight of both the bucks and the does was rather higher in the raw than in the pasteurized group. With the bucks, and with the two sexes taken together, the observed difference was more than three times the standard error of the difference.

Table III. *Intermediate experiments. Synoptic table analysing the detailed results of Exps.* 26–38 *recorded in Table II*

	Raw milk					Pasteurized milk					Raw and pasteurized milk		
	No.		Average initial wt.	Average final wt.	S.D. final wt.	No.		Average initial wt.	Average final wt.	S.D. final wt.	O.D.	S.E. diff.	O.D. / S.E. diff.
	Start	End				Start	End						
Bucks	296	249	14·47	23·92	4·85	299	226	13·76	22·09	6·27	+1·83	0·518	+3·53
Does	298	250	13·92	19·86	4·55	279	229	13·26	18·88	5·07	+0·98	0·442	+2·22
Bucks and does	594	499	14·19	21·88	5·09	578	455	13·52	20·47	6·05	+1·41	0·363	+3·88

S.D. = standard deviation. O.D. = observed difference between the final weight of the mice in the raw and pasteurized groups, making no allowance for the difference in their initial weight (see text). S.E. diff. = standard error of this difference. + means in favour of raw milk.

Before passing on to the breeding results, attention may be drawn to the relatively high mortality of the mice in the growth experiments. In the raw group 16·0 per cent, and in the pasteurized group 21·3 per cent of the animals died during the period of observation. Since none of the deaths was due to demonstrable bacterial infection, this high mortality may probably be attributed to the unsatisfactory nature of the diet.

Breeding performance.

The relevant data are set out in Table IV and analysed in Table V. Little or no difference was noticeable between the raw and pasteurized groups in relation to rapidity of conception, average number of litters produced, or average number of litters reared. On the other hand, as was found in the preliminary experiments (Wilson & Cowell (9)), the average weight of the young animals at the time of weaning was higher in the raw than in the pasteurized

Table IV. *Intermediate experiments. Breeding results of does in Exps.* 27–36, *fed on raw or pasteurized milk supplemented by a mineral mixture, Yeastrel, and a white-flour biscuit. Milk given in inverted tubes.* (*Weights recorded in grams*)

Exp. no.	Group	No. does	Aggregate days from placing with buck to parturition	Litters produced	Litters reared	After 28 days: No. of mice in litters		After 28 days: Aggregate weight of mice in litters	
						Bucks	Does	Bucks	Does
27	Raw	8	350	7	3	7	2	71	18
	Past.	8	164	6	3	8	6	90	65
28	Raw	25	696	23	18	44	57	519	688
	Past.	24	610	21	12	35	26	371	253
29	Raw	29	440	16	12	27	31	290	303
	Past.	29	654	23	15	33	35	319	333
30	Raw	12	281	8	7	15	12	184	150
	Past.	12	246	7	7	20	17	239	194
32	Raw	30	466	18	16	33	42	375	445
	Past.	27	397	15	13	27	34	266	337
33	Raw	11	187	6	6	15	20	162	224
	Past.	12	211	7	7	25	19	276	188
34	Raw	26	313	10	7	21	23	239	255
	Past.	20	343	12	9	23	20	262	213
35	Raw	24	411	10	8	22	20	224	191
	Past.	22	313	8	4	15	9	151	100
36	Raw	18	434	12	8	19	24	182	211
	Past.	13	197	5	3	10	2	103	22

Note. No breeding experiments were carried out with the does of Exps. 26, 37, or 38.

Table V. *Intermediate experiments. Analysis of results of breeding experiments recorded in Table IV*

	Raw	Pasteurized
Total number of does	183	167
Average number of days from placing with buck to parturition	32·53	30·14
Average number of litters per doe	0·60	0·62
Average number of litters reared per doe	0·46	0·44
Average number of bucks per litter reared	2·39	2·68
Average number of does per litter reared	2·72	2·30
Average number of bucks and does per litter reared	5·11	4·99
Average weight in grams of bucks reared	11·06	10·60
Average weight in grams of does reared	10·76	10·15
Average weight in grams of bucks and does reared	10·90	10·39
S.D. of weights of bucks reared	2·02	2·26
S.D. of weights of does reared	2·37	1·97
S.D. of weights of bucks and does reared	2·22	2·15
S.E. of difference between raw and pasteurized mice in weights of bucks reared	0·21	$\frac{\text{O.D.}}{\text{S.E. diff.}} = +2{\cdot}19$
S.E. of difference between raw and pasteurized mice in weights of does reared	0·22	$\frac{\text{O.D.}}{\text{S.E. diff.}} = +2{\cdot}77$
S.E. of difference between raw and pasteurized mice in weights of bucks and does reared	0·15	$\frac{\text{O.D.}}{\text{S.E. diff.}} = +3{\cdot}40$

S.D. = standard deviation. O.D. = observed difference. S.E. diff. = standard error of difference. + means in favour of raw milk.

group. The difference is barely significant when the bucks and does are considered separately, but when they are taken together the observed difference is 3·40 times its standard error.

Final experiments, Nos. 39–49

In both the preliminary and the intermediate experiments there was one serious technical error, the importance of which became more and more obvious as our observations increased. Though every attempt was made to render the comparison between the raw and pasteurized groups as perfect as possible, the way in which the milk had to be given—namely, in inverted tubes—frustrated this endeavour. In the tubes containing raw milk the cream rose rapidly to the top, so that the animals sucking from the lower end obtained milk with a low fat content. On the other hand, in the tubes containing pasteurized milk, the cream layer at the top was considerably less, with the result that the animals in the pasteurized group obtained milk with a higher fat content than in the raw group. Repeated observations showed that the average amount of milk consumed by the animals in the raw group was greater than that in the pasteurized group, and it was therefore suspected that the fat content of the pasteurized milk was too high for the digestive organs of the normal mouse. Unfortunately, we were unable to obtain any figures for the average fat content of mouse's milk, so that this suspicion could not be directly confirmed. It seemed important, however, to find out whether the difference in the available fat content of raw and pasteurized milk when given in tubes was likely to be responsible for the observed differences in rate of growth.

Two methods were tried. In Exps. 44 and 45 the milk was centrifuged, the fat removed, and the separated milk given in inverted tubes. The difference in fat content between the separated raw and pasteurized milk was negligible. In Exps. 39–43 and again in Exps. 46–49, whole milk was given in open drinking vessels instead of in inverted tubes. Since a larger amount of cream rose to the surface in the raw than in the pasteurized milk, it followed that the mice in the raw group tended to drink milk with a rather higher fat content than in the pasteurized group. It was thought that, if excessive fat did cause digestive disturbances, the growth of the mice on raw milk should be less satisfactory than on pasteurized. As will be seen presently, this result was actually obtained.

The mice in Exps. 39–41 had all been raised by does on a raw or pasteurized milk diet, supplemented by mineral mixture, Yeastrel, and biscuit, and represented second or third generation mice on a raw or pasteurized milk diet. Towards the conclusion of these experiments a Salmonella infection appeared in the breeding mice, and in order not to invalidate the results of a nutrition experiment by working with latently infected animals, the whole breeding stock surviving from the intermediate experiments was killed off. The re-

maining experiments, 42–49, had therefore to be made on litters brought up in the normal animal house by does fed on a stock diet. This consisted of a mixture of oatmeal, bran, Yeastrel, cod-liver oil, and diluted pasteurized milk. All animals were weaned at 4 weeks and transferred to the usual diet of raw or pasteurized milk supplemented by a mineral mixture and a white-flour biscuit.

Results of final experiments

Owing to pressure of other work it proved impossible to carry out breeding experiments after the conclusion of the observations on growth. The results, therefore, are concerned solely with rate of growth.

Rate of growth in separated milk experiments.

Two experiments were performed, Nos. 44 and 45. They are included with the remainder of the final experiments in Table VI.

It will be seen that in both experiments the final weight of the mice was greater in the pasteurized than in the raw group, and that the difference in weight between the two groups was greater with the bucks than with the does. The results are analysed in Table VII.

The numbers of mice used were unfortunately too small to permit of significant differences being obtained. Nevertheless the observed difference for both sexes divided by its standard error was as high as 1·61.

Rate of growth in open-vessel milk experiments.

By an oversight the weights of the individual mice at the close of observation were not recorded in Exps. 39–43; only the aggregate weights of the mice are available. In calculating the standard error of the difference between the raw and pasteurized groups, the mean standard deviation observed in Exps. 44–49 was therefore adopted for the whole series.

The results, which are recorded in detail in Table VI and analysed in Table VIII, are seen to be different from those obtained in the intermediate experiments.

The bucks in the raw group were rather heavier at the end of the experiment than in the pasteurized group, while the does in the pasteurized group were heavier than those in the raw group. Taking both sexes together the final weight of the animals was rather greater in the pasteurized than in the raw group, though the observed difference was well below the limit of significance. In this connexion, however, it must be noted that the total number of mice used was only about half that in the intermediate experiments. If the same number of mice had been used, it is possible that the difference might have been more striking. It must also be noted that the comparison was not perfectly fair to the pasteurized group. Though during the week the milk in Exps. 39–43 and 46–49 was given in open vessels, on Saturdays and Sundays it was given in inverted tubes. On these days the milk could be given only

Table VI. *Final experiments. Growth of mice on a diet of raw or pasteurized milk supplemented by a mineral mixture and a white-flour biscuit. Whole milk given in open vessels on 5 days a week, and in inverted tubes during the week-end, in Exps. 39–43 and 46–49; separated milk given in inverted tubes in Exps. 44 and 45. (Weights recorded in grams)*

			Bucks					Does					
			No.					No.					
Exp. no.	Length of exp. in weeks	Group	Start	End	Average initial wt.	Average final wt.	S.D. final wt.	Start	End	Average initial wt.	Average final wt.	S.D. final wt.	Remarks
39	11	Raw	26	21	16·54	21·23	—	31	25	15·38	18·04	—	Litters from does fed on raw or pasteurized milk in Exp. 33
		Past.	34	27	17·02	20·48	—	29	25	16·14	17·56	—	
40	14	Raw	12	2	15·00	25·50	—	18	8	13·77	16·25	—	Ditto in Exp. 34
		Past.	13	7	16·07	20·00	—	8	3	15·62	24·00	—	
41	13	Raw	32	16	12·43	16·44	—	34	23	12·00	16·04	—	Ditto in Exp. 35
		Past.	20	17	12·70	17·94	—	9	6	14·44	18·17	—	
42	12	Raw	23	20	13·56	19·60	—	23	19	12·70	17·58	—	Litters from does fed on stock diet
		Past.	16	8	12·78	17·50	—	23	15	11·96	16·20	—	
43	12	Raw	8	7	12·37	24·43	—	9	8	11·88	21·88	—	,,
		Past.	7	6	11·57	23·00	—	6	5	12·83	21·40	—	
44	13	Raw	14	8	12·64	24·50	4·85	11	8	13·09	25·50	2·06	,,
		Past.	14	8	13·21	29·00	4·42	11	7	13·64	27·14	1·51	
45	12	Raw	12	7	12·92	25·29	3·02	14	11	12·57	25·64	2·31	,,
		Past.	11	6	12·91	26·67	4·36	13	9	13·00	24·67	4·45	
46	13	Raw	21	19	13·90	27·37	3·59	15	13	13·87	26·38	3·09	,,
		Past.	21	19	13·81	27·64	3·72	16	15	13·31	25·86	3·28	
47	12	Raw	16	15	11·88	30·73	4·07	13	13	13·62	28·00	3·64	,,
		Past.	10	6	12·60	31·17	2·22	17	16	11·82	25·56	2·89	
48	12	Raw	21	17	9·75	29·71	3·02	11	11	11·75	25·64	2·35	,,
		Past.	15	15	8·87	31·87	3·15	7	7	8·14	26·29	2·97	
49	12	Raw	10	10	15·80	30·20	3·71	15	14	13·00	25·07	2·54	,,
		Past.	10	8	14·40	25·50	3·64	15	14	13·47	26·57	3·77	

S.D. = Standard deviation.

Table VII. *Final experiments. Synoptic table analysing the detailed results of Exps.* 44 *and* 45 *recorded in Table VI. Separated milk given in inverted tubes*

	Raw milk					Pasteurized milk					Raw and pasteurized milk		
	No.		Average initial wt.	Average final wt.	S.D. final wt.	No.		Average initial wt.	Average final wt.	S.D. final wt.	O.D.	S.E. diff.	O.D. / S.E. diff.
	Start	End				Start	End						
Bucks	26	15	12·77	24·87	4·11	25	14	13·08	28·00	4·55	−3·13	1·62	−1·93
Does	25	19	12·80	25·58	2·21	24	16	13·29	25·75	3·70	−0·17	1·06	−0·16
Bucks and does	51	34	12·78	25·26	3·26	49	30	13·18	26·80	4·27	−1·54	0·96	−1·61

S.D. = standard deviation. O.D. = observed difference between the final weight of the mice in the raw and pasteurized groups, making no allowance for the difference in their initial weight. S.E. diff. = standard error of this difference. − means in favour of pasteurized milk.

Table VIII. *Final experiments. Synoptic table analysing the detailed results of Exps.* 39–43 *and* 46–49 *recorded in Table VI. Whole milk given in open vessels on* 5 *days a week and in inverted tubes during the week-end*

	Raw milk					Pasteurized milk					Raw and pasteurized milk		
	No.		Average initial wt.	Average final wt.	S.D. final wt.	No.		Average initial wt.	Average final wt.	S.D. final wt.	O.D.	S.E. diff.	O.D. / S.E. diff.
	Start	End				Start	End						
Bucks	169	127	13·40	24·50	3·85	146	113	13·96	23·62	4·21	+0·88	0·529	+1·66
Does	169	134	13·26	20·89	3·19	130	106	13·45	21·92	3·29	−1·03	0·424	−2·43
Bucks and does	338	261	13·33	22·64	3·85	276	219	13·72	22·79	4·05	−0·15	0·361	−0·42

S.D. = standard deviation. O.D. = observed difference between the final weight of the mice in the raw and pasteurized groups, making no allowance for the difference in their initial weight. S.E. diff. = standard error of this difference. + means in favour of raw milk. − means in favour of pasteurized milk.

Note. The standard deviation for Exps. 39–43 was taken as being equal to that for Exps. 44–49 (see text).

once instead of twice a day, since it was found that in open vessels the raw milk was usually bad by evening. For this reason the inverted-tube method was adopted for the week-ends. If, as seems probable, the inverted-tube method favoured the raw mice and the open-vessel method the pasteurized mice, it is clear that the practice of supplying milk in open vessels on only 5 days a week was likely to reveal a smaller difference between the two groups than if it had proved possible to use it continuously.

As in the intermediate experiments, a considerable mortality was observed among the mice. In the whole series of final experiments 24·2 per cent of animals in the raw, and 23·4 per cent in the pasteurized group died. Bacteriological examination failed to reveal any specific infection among the animals, and it was therefore concluded that the imperfect nature of the diet was responsible.

Summary and conclusions

1. Experiments were carried out during the years 1931–5 in which mice on a basal diet of raw milk and white-flour biscuit were compared with mice on a similar diet except that the raw milk was replaced by commercially holder-pasteurized (145–150° F. for 30 min.) milk belonging to the same batch. Each

group of mice comprised in aggregate about 1100 animals, which were usually 4–6 weeks old at the commencement of observation. For reasons given on p. 206 no attempt was made to standardize the volume of milk consumed by the individual mice. Except in the preliminary experiments, when the milk was given in excess, the amount supplied was regulated more or less in accordance with the requirements of the average animal.

2. Preliminary experiments, Nos. 1–25, which have already been reported by Wilson & Cowell(9), showed that both types of milk were quite inadequate to promote normal growth, and that young mice on this dietary generally died within a few weeks in an emaciated and often severely anaemic condition. A supplement of iron, copper and manganese improved growth and development and prevented anaemia, but did not usually suffice to enable breeding does to rear their litters. The addition of Yeastrel overcame this difficulty, and when both mineral mixture and Yeastrel were added, growth, reproduction, and rearing of the litters appeared to be almost as satisfactory as on a simple stock diet of oats and dilute boiled milk. It is concluded that, so far as mice are concerned, cow's milk is deficient in mineral substances, and in some factor, possibly vitamin B, which is required for successful lactation.

3. In the preliminary experiments little difference was observed between the raw and pasteurized groups in respect of general development, mortality, production of litters, or successful rearing of the young. With mice brought up from birth, however, by does on a milk-and-biscuit diet, supplemented by mineral mixture and Yeastrel, the average weight of the young mice at weaning time (4 weeks) was significantly greater in the raw than in the pasteurized groups.

4. In the intermediate group of experiments, Nos. 26–38, the milk-and-biscuit diet was uniformly supplemented by a mineral mixture. In addition the breeding bucks at the time of mating, and the breeding does from the time of mating to the time of weaning, were given Yeastrel. Growth was observed in different experiments from 7 to 19 weeks, after which the does were mated and their breeding performance studied. The general results of the intermediate experiments confirmed those of the preliminary experiments, but a significant difference was found in favour of the raw group in the weight of the mice in the growth experiments, as well as in the weight of the young mice at weaning time in the breeding experiments.

5. In the final experiments, Nos. 39–49, the milk was given 5 days a week in open vessels instead of in inverted tubes as in the preliminary and intermediate experiments. In two experiments, Nos. 44 and 45, separated milk was given in inverted tubes. The results were different from those previously obtained. The pasteurized mice at the conclusion of the experiments were rather heavier than the raw, though the difference in weight between the two groups was not significant. Unfortunately, pressure of other work prevented the carrying out of breeding experiments with does given milk in open vessels.

6. The method by which the milk was given appeared to have a marked

influence on the result. When it was given in inverted tubes, the raw mice did better; when it was given in open vessels the pasteurized mice did better. It was originally thought possible that this might be due to differences in the fat content of the milk ingested by the mice under the two sets of conditions. Thus, when the milk was given in inverted tubes more cream rose to the top in raw than in pasteurized milk, so that the mice sucking from the lower end obtained less fat in the raw than in the pasteurized group. On the other hand, with milk in open vessels, the mice in the raw group ingested more cream than in the pasteurized group. The suggestion was that the fat content of cow's milk was too high for the digestive functions of the mouse. It is very doubtful, however, whether this is the correct explanation. For one reason, it does not explain why, in the experiments in which separated milk was given, the average increase in weight was so much higher in the pasteurized than in the raw group. Theoretically, the two groups ought to have behaved alike. Another reason is that the analyses of rats' milk recently published by Cox & Mueller(11) show that the fat content is very much higher (14·8 per cent) in rat's than in cow's milk (3·4 per cent). Unless mouse's milk, for which no analyses are available, differs fundamentally in composition from rat's milk, it seems unlikely that the digestive functions of the mouse should be overtaxed by the amount of fat in cow's milk. It is possible that variations in the quantity of milk drunk under the different sets of conditions may have been responsible for the differences observed in the growth rate of mice fed by the inverted-tube and by the open-vessel methods, but unfortunately sufficient exact observations were not made on this point. Whatever the real explanation may be, the observation that assessment of the nutritive value of raw and pasteurized milk depends so much on the actual technique used is itself a fact of considerable importance.

7. It may be noted that in the intermediate and final experiments, second and third generation mice on a milk-and-biscuit diet appeared after weaning to grow as rapidly as those brought up on a simple stock diet. No evidence of progressive deterioration was observed in successive generations.

8. Owing to the importance of the method by which the milk is given in determining the results of milk-feeding experiments, and owing to the fact that the majority of our observations were made with the inverted-tube method, which has been shown to favour the raw mice, it is impossible to draw any definite conclusions from our experiments on the relative nutritive value for mice of raw and pasteurized milk. If there is a difference, it must be extremely slight, and no comparative experiment to determine it, carried out on lines similar to those employed in the present work, would probably be worth while making on less than five hundred to a thousand mice in each group.

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159. THE RESISTANCE TO INFECTION WITH *BACT. TYPHI-MURIUM* OF MICE FED ON RAW AND PASTEURIZED MILK

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In previous papers (Wilson & Cowell(1); Wilson & Maier(2)) a description has been given of the growth and breeding behaviour of mice fed on a dietary whose main constituent was raw or pasteurized milk. In the present paper an account is given of the reaction of the animals to infection with a natural parasite of the mouse—*Bact. typhi-murium.*

Description of experiments

The mice used were taken from Exps. 26–38 and 44–49, described in the paper by Wilson & Maier(2). The animals in the first group of experiments had been fed on raw or pasteurized whole milk in inverted tubes, supplemented by a mineral mixture of iron, copper, and manganese and a biscuit made of white flour and water. In addition the does had received 3 per cent of Yeastrel during the breeding time. The mice in Exps. 44 and 45 had received separated milk in inverted tubes with the usual supplements, whereas those in Exps. 46–49 had been given whole milk in open vessels—again with the usual supplements. So far as growth was concerned, the method of feeding was found to be of considerable importance. When whole milk was given in inverted tubes, the animals in the "raw" group did better; when it was given in open vessels, those in the "pasteurized" group did better. It was considered important therefore, when carrying out the virulence tests, to keep mice that had been fed in one way separate from those that had been fed in a different way. In some experiments both bucks and does from any given feeding experiment were tested at the same time, but more usually the bucks were tested at the conclusion of the growth experiment, and the does after they had weaned their litters. The distribution of the mice is indicated in the second column of Table I.

Two sets of experiments were made. In one set the animals were inoculated intraperitoneally with a dose of about 100 or 1000 living organisms, and observed for 2 weeks; in the other set they were fed by the mouth with about 100–200 million organisms and observed for 4 weeks. All animals dying during the course of the experiment, and all survivors killed at the end, were submitted to a bacteriological post-mortem examination to determine whether they were infected with the specific organism.

The strain of *Bact. typhi-murium* used was of fairly high virulence. It was kept in stab agar and was subcultured once a month. When a virulence test was to be made, the strain was subcultured into heart extract broth, and

grow for 18–24 hours at 37° C. The culture was then diluted to the required degree with Ringer's solution. For intraperitoneal inoculation 0·5 ml. of the diluted culture was used; for feeding, two drops were given from a dropping pipette delivering 200 drops per ml. A viable count was made by the roll-tube method on the actual dilution used for inoculation. Raw and pasteurized mice were inoculated alternately.

After inoculation each mouse was transferred to a separate cage of the pill-box type and fed on a diet of raw or pasteurized whole milk, supplemented by mineral mixture and white-flour biscuit. All milk was given in inverted tubes, irrespective of the mode of feeding before inoculation.

RESULTS

The results are given in Table I, and the more important data are summarized in Table II.

Taking Exps. 1–5 together, it will be noted that the proportion of specific deaths and of total infected mice was less in the pasteurized than in the raw group, but on the other hand, the average survival time of mice dying of specific infection was longer in the raw group. These results were determined to some extent by the weight of the animals in the different groups. The average weight of the pasteurized mice was less than that of the raw. Examination of the data showed that among all mice of 20 g. or less the specific mortality was 69·1 per cent, whereas among all mice of over 20 g. it was only 45·2 per cent. It was thought worth while to work out specific death-rates in the raw and pasteurized groups standardized for weight distribution. The resulting figures were 55·1 per cent for the raw and 50·6 per cent for the pasteurized group. The standard error of this difference was 5·3, and the observed difference divided by its standard error was 0·85. Clearly none of the differences observed between the two groups can be regarded as significant.

In Exps. 6–9 the mice in the raw-milk group had some advantage as regards specific mortality and average survival time, but not as regards the total number infected.

In Exps. 10–12 the proportion of specific deaths and of total infected was less in the raw than in the pasteurized group, but the average survival time of mice dying of specific infection was longer in the pasteurized group. It may be noted that a considerable number of non-specific deaths occurred in both groups. One probable reason for this was that after infection a change was made either in the composition of the dietary or in the mode of its administration. In Exp. 10 separated milk was replaced by whole milk, and in Exps. 11 and 12 milk was given in inverted tubes instead of in open vessels. Changes of this sort, particularly when mice are kept in single cages, sometimes react on the well-being of the animals. Other causes were probably operative, however, since a high non-specific death-rate was observed in some of the other experiments.

Table I. *Results of inoculation of mice, by intraperitoneal injection or by feeding, with* Bact. typhi-murium

Virulence exp.	Origin of mice	Diet before inoculation	Mode of infection	Dose of living organisms	Raw					Pasteurized				
					No.	Deaths	Sp.D.	Total inf.	A.S.T. Sp.D.	No.	Deaths	Sp.D.	Total inf.	A.S.T. Sp.D.
1	B.D. 26	Whole milk in inverted tubes	Ip.	170	60	27	26	56	6·69	53	38	27	39	4·56
2	B. 27, 28		,,	90	37	17	17	37	7·82	24	9	9	24	5·22
3	B. 29		,,	120	26	11	10	23	8·70	24	11	11	24	9·36
4	D. 27–29, B. 30		,,	900	32	27	26	30	9·00	33	21	21	33	8·14
5	D. 27–29		,,	1500	36	20	20	36	8·50	32	17	17	32	8·53
Total					191	102	99	182	8·06	166	96	85	152	6·93
6	D. 27–29, B. 32	Whole milk in inverted tubes	Feeding	80,000,000	34	11	8	23	18·25	37	21	12	21	15·75
7	D. 30, B. 33, 34			90,000,000	33	14	11	25	14·82	44	25	18	32	14·72
8	D. 32, B. 35, 36			80,000,000	68	26	22	51	14·18	54	28	20	40	15·55
9	D. 33–36, B. 37, 38			80,000,000	79	29	27	69	13·52	66	34	30	52	12·43
Total					214	80	68	168	14·50	201	108	80	145	14·23
10	B.D. 44, 45	Separated milk in inverted tubes	Feeding	250,000,000	34	32	16	16	13·88	30	28	21	21	12·19
11	B.D. 46, 47	Whole milk in open vessels		250,000,000	60	46	40	46	12·60	56	34	29	35	14·72
12	B.D. 48, 49			190,000,000	52	43	13	13	15·31	42	31	16	16	14·75
Total					146	121	69	75	13·41	128	93	66	72	13·92
6–12	Total		Feeding		360	201	137	243	13·95	329	201	146	217	14·09

B. = bucks. D. = does. Ip. = intraperitoneal. Sp.D. = specific deaths. Total inf. = total number of mice dying or killed from which the specific organism was isolated. A.S.T. Sp.D. = average survival time in days, limited to 14 or 28 days, of the animals dying of specific infection.

Table II. *Analysis of Table I*

Virulence exp.	Diet before inoculation	Mode of infection	Comparison of	O.D.	S.E. diff.	O.D. / S.E. diff.
1–5	Whole milk in inverted tubes	Intraperitoneal	Specific deaths	−0·63%	5·30	−0·12
			Total infected	−3·72%	2·60	−1·43
			A.S.T. of mice dying of specific infection	+1·13 days	0·49	+2·31
6–9	Whole milk in inverted tubes	Feeding	Specific deaths	+8·02%	4·71	+1·70
			Total infected	−6·36%	4·23	−1·50
			A.S.T. of mice dying of specific infection	+0·27 days	0·88	+0·31
10–12	Separated milk in inverted tubes or whole milk in open vessels	Feeding	Specific deaths	+4·30%	6·05	+0·71
			Total infected	+4·88%	6·04	+0·81
			A.S.T. of mice dying of specific infection	−0·51 days	0·83	−0·61
6–12	Whole milk in inverted tubes or open vessels; or separated milk in inverted tubes	Feeding	Specific deaths	+6·32%	3·75	+1·69
			Total infected	−1·54%	3·59	−0·43
			A.S.T. of mice dying of specific infection	−0·14 days	0·61	−0·23

A.S.T. = average survival time.
O.D. = observed difference between raw and pasteurized mice.
S.E. diff. = standard error of this difference.
+ means in favour of raw milk mice.
− means in favour of pasteurized milk mice.

Taking all feeding experiments together, Exps. 6–12, it will be noted that the advantage lay with the raw mice as regards the proportion of specific deaths, but with the pasteurized mice as regards the proportion of total infected, and the average survival time of mice dying of specific infection.

Examination of the data showed that in the feeding experiments the weight of the animals made very little difference. Of all mice of 20 g. or under the specific mortality was 44·1 per cent, and of all mice over 20 g. it was 40·6 per cent. The death-rates standardized for weight distribution were 38·4 per cent for the raw and 44·6 per cent for the pasteurized mice. The standard error of this difference was 3·76, and the observed difference divided by its standard error was 1·65.

DISCUSSION

These results require little discussion. The differences between the raw and pasteurized groups were never large and never approached significance. Long experience has shown us that differences as great as, and greater than, these are quite often observed between two groups of mice belonging to the same batch and treated under identical conditions. The differences, moreover, were not constant; they fluctuated from one group of experiments to another. For example, in the mice inoculated intraperitoneally the proportion of specific deaths was higher in the raw group, and in the mice fed by the mouth it was

higher in the pasteurized group. On the other hand, the average survival times of the animals dying of specific infection were in the reverse order.

It may be noted that all mice after inoculation were given milk in inverted tubes. Since our previous observations indicated that this method of feeding favoured the development of mice in the raw group, it was obviously weighting the odds slightly against the pasteurized mice in Exps. 11 and 12 to substitute this method for that of feeding in open vessels. The latter method, however, could not have been satisfactorily employed for infected mice; had it proved practicable, the results in these two experiments might have been more favourable to the pasteurized group. If all the mice in all the experiments previous and subsequent to inoculation had been fed on milk in open vessels the results might have been even more favourable to the pasteurized animals. This, however, is mere surmise. The real truth is that there is no conclusive evidence to show that the specific resistance of the animals in these experiments was related to the type of diet they were receiving.

Attention should perhaps be drawn to the recent results of Watson (3), who found that feeding afforded a more delicate index of resistance to infection with *Bact. typhi-murium* than intraperitoneal inoculation. The two methods, however, yielded similar results, and differed only in degree. In our experiments the two methods gave opposite results, and with neither method were the results significant. It does not seem probable, therefore, that they were determined to any considerable extent by differences in resistance due to dietetic factors.

Summary and conclusions

1. In a series of virulence experiments a comparison was made between the resistance to infection of 551 mice fed on raw and 495 mice fed on pasteurized milk, each dietary being supplemented by a mineral mixture and a white-flour biscuit. In one set of experiments the mice were inoculated intraperitoneally with about 100 or 1000 living *Bact. typhi-murium* and observed for a fortnight; in another set they were fed by the mouth with about 100–200 million living organisms of the same strain and observed for 4 weeks. All animals dying during, and all survivors killed at the end of the experiments were bacteriologically examined for evidence of specific infection.

2. In the intraperitoneal inoculation experiments the results obtained with the raw and pasteurized groups respectively were as follows: Proportion of animals dying of specific infection 51·83 and 51·20 per cent; proportion of all animals found to be specifically infected at death or when killed 95·29 and 91·57 per cent; average survival time of animals dying of specific infection 8·06 and 6·93 days. The proportions of animals dying of specific infection when standardized for weight distribution were 55·1 and 50·6 per cent respectively.

3. In the feeding experiments the results obtained with the raw and pasteurized groups respectively were as follows: Proportion of animals dying of specific infection 38·06 and 44·38 per cent; proportion of all animals found

to be specifically infected at death or when killed 67·50 and 65·96 per cent; average survival time of animals dying of specific infection 13·95 and 14·09 days. The proportions of animals dying of specific infection when standardized for weight distribution were 38·4 and 44·6 per cent respectively.

4. With one exception, none of the observed differences between the raw and pasteurized groups in respect of any of the three factors analysed approached even twice its standard error. Some of the differences were in favour of the raw mice, others in favour of the pasteurized mice. Differences as great as, or even greater than, those observed are often met with between two groups of mice of the same batch kept under identical conditions.

5. There is no evidence that mice fed on pasteurized milk differ from those fed on raw milk in their resistance to infection with a natural parasite such as *Bact. typhi-murium*.

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160. LACTIC BACTERIA IN RELATION TO CHEESE FLAVOUR. I

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The origin of flavour in Cheddar cheese is still obscure, despite the considerable amount of experimental work upon cheese-ripening which has been carried out during the last 50 years.

The early investigations scarcely call for attention here. A good deal of interest attaches, however, to the later work of Hastings *et al.*(1) who, apparently, were the first to delineate the sequence in bacterial flora which is now generally accepted as occurring during the process of normal Cheddar cheese-ripening, viz. *B. lactis acidi* (*S. lactis* and *S. cremoris*) in the early stages, replaced, after conversion of the lactose to acid, by *B. casei* and occasionally large numbers of cocci. The work of these authors indicated that particular groups of *B. lactis acidi* contributed to the characteristic flavour of cheese, while certain strains of *B. casei* appeared to be largely responsible for the pungent flavour which develops in well-matured cheese(2). In more recent investigations Evans devoted her attention mainly to the influence of streptococci upon flavour formation. She found that when a mixed starter consisting of *B. lactis acidi* and a certain *Streptococcus* (probably a *Betacoccus*) was employed in cheese-making experiments, the flavour of the cheese was improved(3). Although in many instances the milk used had been pasteurized, large numbers of *B. casei* always made their appearance in the cheese. These studies led Evans to the conclusion that streptococci were capable of modifying cheese flavour to a significant extent.

Harrison & Connell(4), on the other hand, considered that the most important bacteria in relation to flavour development in Cheddar cheese were the group of lactobacilli. Burri & Staub(5) ascribed to *B. casei* Freudenreich the paramount role in the ripening of Emmental cheese.

Hucker & Marquardt(6) examined Cheddar cheese which had been manufactured with the use of strains of *S. paracitrovorus* as starter, and they advanced the opinion that these organisms were capable of directly influencing cheese flavour. Their deductions, however, were drawn from the study of comparatively few small cheeses, and apparently no attempts were made to determine whether the added organisms flourished in the cheeses. Moreover, it is clear that the use of various strains of starter organisms which formed acid (and other products) at different rates might lead to the production of cheese curds which varied in chemical composition. Apart from the direct effect upon cheese flavour of the varying amounts of acid present, the different compositions of the green cheeses would influence subsequent changes, in-

cluding flavour formation, brought about by bacterial action. Further, it is quite conceivable that these chemical differences in the cheeses might even selectively favour the growth from the same milk flora of *different* strains of flavour-forming organisms.

It seems to the writer that many investigators, who have inoculated the milk employed in cheese manufacture with lactic bacteria, have given insufficient attention to the desirability of demonstrating the presence of these added organisms in the resultant cheeses. The development of cheese flavour is a slow process, and unless the organisms inoculated (especially if they were isolated from sources other than cheese) have been shown to be capable of good growth in cheese, it is unsafe to ascribe to them any direct effect upon flavour production.

Data collected in this Institute over a number of years have failed to support the view that different pure strains of starter streptococci are specifically capable of producing characteristic, desirable flavours in cheese. Furthermore, typical Cheddar flavour does not usually appear during the life of the starter streptococci which, in general, die out very early in the ripening period. Cheese, apparently, is an unsuitable medium for their proliferation. Further evidence in support of this is given by the work of Hucker(7), who reports that starter organisms do not appear to form colonies in cheese. Consequently, it is to be expected that if any flavours are developed by the streptococci of this group such flavours will make their appearance early in the green cheese. This view is borne out by practical experience, e.g. during the 1931 season one starter employed in the Institute was found to impart to cheese a distinct bitterness(8); this objectionable flavour was readily detected in the cheese a fortnight after manufacture (the first examination) and persisted throughout maturation.

The possibility that rennet enzymes play a direct part in flavour formation has not received support from recent work(9).

It has been a common observation, however, that the flora of locally manufactured cheese usually exhibits, after a ripening period which varies from several days to a few weeks, a very high proportion of lactobacilli. The incidence of these organisms before typical Cheddar flavour makes its appearance suggests an important rôle for the group of lactobacilli in the development of flavour.

This paper describes two series of cheese-making experiments in which the milk employed was inoculated with strains of lactobacilli.

Series I. The inoculation of cheese-milk with natural mixed cultures of lactobacilli obtained from cheese

The basic idea of this series of experiments, which was spread over an interval of approximately 12 months, was that if lactobacilli are responsible for the development of cheese flavour it might be possible to reproduce

desirable flavours in cheese by inoculation of the cheese-milk with a natural mixture of lactobacilli from a good quality cheese. Similarly, the addition to the cheese-milk of a natural mixture of organisms from poor quality cheese should lead to the development of "off" flavour in the resultant cheese.

Experimental

Preparation of cultures.

About 5 g. of each cheese (good quality or poor quality) were emulsified with warm water and an amount corresponding to 0·00001 g. of cheese was added to 350 ml. sterile milk (autoclaved at 12 lb.) containing 5 per cent of yeast extract. The cultures were incubated at 31° C. until clotted (usually 1–2 days).

Method of cheese manufacture.

The milk employed was obtained from the Massey Agricultural College herd and was of good quality (plate counts usually $<100{,}000$/ml. and *coli* absent in 0·001 ml.). In accordance with standard procedure, the milk was flash-pasteurized at 155° F. At this stage plate counts were usually <5000/ml. and *coli* absent in 1 ml.

Two vats were employed, one serving for the manufacture of control cheeses and the other for the inoculated cheeses. To each vat of milk on any one day, the same percentage (approximately $1\frac{1}{4}$ per cent) of the same starter was added, and the manufacturing procedure was kept as closely as possible the same for both vats. To vat 2 the inoculum (0·1–0·2 per cent yeast milk culture) was added just before the addition of rennet. It was thought that this procedure was preferable to addition at an earlier stage, since it minimized acid formation by the added lactobacilli during the manufacturing process. In practice, however, it was found that these organisms did not produce any detectable amount of acid in the vat. The cheeses made were all of medium or export size (40 or 80 lb. respectively) and were ripened in curing rooms for 3 weeks at a temperature of 60° F. and subsequently at 45° F.

In preliminary experiments both control and experimental vats were housed in the same room. Examination of the resulting cheeses when mature indicated clearly that the control vat had, despite all precautions, frequently become contaminated with organisms from vat 2. The results of these experiments, therefore, were discarded. The inoculation vat was removed to a separate room and the inoculated cheeses were manufactured by an isolated cheese-maker. All possible care was taken to avoid contamination of the control vat, separate supplies of rennet, annatto, salt, etc., being kept in the "isolation room". Even with these precautions it was found that, after a manufacturing period of several months, it became difficult to prevent organisms from the "isolation" room being spread through the factory.

Examination of cheeses.

The cheeses were graded at intervals by experienced graders on the Institute Staff. A number of the cheeses were also examined at maturity by members of the Dairy Division of the Department of Agriculture.

The grading remarks which follow represent the consensus of opinion among the graders.

It will be observed that the inoculation of natural mixed cultures from mature cheeses, whether of good or poor quality, in every instance produced unclean, fermented flavours. These undesirable flavours made their appearance earlier and were more intense in the cheeses inoculated with cultures from poor quality cheeses. It is evident from the table that culture 3 N, an inoculum from an *immature* cheese of good quality, did not form a very undesirable flavour. The results obtained in this series will be discussed at greater length when series II is under consideration.

SERIES II. THE INOCULATION OF CHEESE-MILK WITH PURE STRAINS OF LACTOBACILLI ISOLATED FROM CHEESE

In this series of experiments single pure strains or mixtures of several pure strains of lactobacilli and cocci isolated from Cheddar cheese were added to vat 2. The organisms were cultured in milk containing 5 per cent yeast extract. The manufacturing procedure, curing, grading methods etc. were the same as for series I.

The presence of the inoculated strains in the mature cheeses was demonstrated by the isolation of organisms and examination of the following characteristics: colony form on yeast-whey agar, volatile acid formation in yeast-milk (both in the presence and absence of citric acid) and clotting power in milk. In the table which follows, for reasons of space, only representative experiments are cited. The number of cheeses actually examined runs into several hundreds.

It is clear, from Table II, that the addition to cheese-milk of different strains of lactobacilli led to the development of a variety of flavours, both good and bad, in the resultant cheeses. A definitely characteristic flavour appeared to be produced consistently by each organism, or mixture of organisms.

The implications of Table II will be discussed fully, together with the results of series I, in a later section.

Biochemical reactions of the organisms

In the following two tables are recorded certain biochemical characteristics (including sugar reactions) of the 12 pure cultures mentioned in the preceding table.

The methods employed for estimation of the volatile acid and carbon dioxide produced by the organisms in yeast-milk culture were as follows:

Table I. *The effect upon cheese flavour of the inoculation of the milk with natural mixed cultures of lactobacilli obtained from cheese*

F = finest, 1 = 1st grade, 2 = 2nd grade

Cultures employed			Cheeses resulting			
				Remarks on flavour and the grade of cheese at different ages		
Culture no.	Source of culture	% inoculum	Cheese	4 weeks	6 weeks	17 weeks
1 N	Poor quality mature cheese	0·10	Control	Very good, F	Very good, F	Very good, F
			Inoculated	Fermented, 2	Strong, fermented, 2	Strong, fermented, 2
2 N	Very good quality mature cheese	0·10	Control	Very good, F	Very good, F	A little acid, 1
			Inoculated	Winey, 2	Unclean, 2	Strong, fermented, 2
3 N	Very good quality immature cheese	0·10	Control	Not quite clean, 1	Sharp, clean, 1	Mild, mature, 1
			Inoculated	Sour, fermented, 1	Fermented, 2	Slightly saline, 1
4 N	Very good quality mature cheese	0·05	Control	Fair, 1	Good, 1	Mild, mature, 1
			Inoculated	Very fermented, 2	Very fermented, 2	Strong, fermented, 2
4 N	—	0·10	Control	Clean acid, F	Slightly unclean, 1	Mature, 1
			Inoculated	Sharp, inclined bitter, 1	Sharp, fermented, 2	Sharp, fermented, 2
5 N	Poor quality mature cheese	0·10	Control	Clean, F	Sharp, 1	Clean, mature, F
			Inoculated	Strong, fermented, 1	Strong, putrefactive, 2	Pungent, fermented, 2
6 N	Poor quality immature cheese (raw-milk)	0·20	Control	Clean, good, F	—	Sharp, acid, 1
			Inoculated	Unclean, 2	—	Sweet, fermented, 2
6 N	—	0·20	Control	Very good, F	—	Clean, 1
			Inoculated	Unclean, 2	—	Very unclean, 2

Table II. *The effect upon cheese flavour of inoculation of the cheese-milk with pure strains of lactobacilli*

F = finest, 1 = 1st grade, 2 = 2nd grade

Cultures employed				Cheeses resulting				
					Remarks on flavour and the grade of cheese at different ages			
Culture no.	Source of culture	Appearance of organisms in milk culture	% inoculum	Cheese no.	2 weeks	4 weeks	6 weeks	17 weeks
1	Good quality mature cheese	Bacilli (very small; short chains)	0·10	Control, 1	—	Good, clean, F	Clean, 1	Clean, mature, F
				Inoculated, 1	—	Fair, 1	Clean, mature, 1	Slightly better than control, F
1	—	—	0·10	Control, 2	—	Clean, 1	Clean, good, 1	Good, clean, F
				Inoculated, 2	—	Sharp, clean, 1	Good, little sharp, 1	Good, mature, F
2*	Good quality mature cheese	Bacilli (short, thick; short chains)	0·10	Control, 3	—	Sharp, acid, 1	Sharp, acid, 2	Clean, acid, 1
				Inoculated, 3	—	Sharp, aromatic, 1	Better than control, 1	Aromatic, 1
3	Average quality immature cheese	Bacilli (short, thick; short chains)	0·10	Control, 4	Clean, good, F	—	—	Good, a little acid, F
				Inoculated, 4	Acetic acid, 1	—	—	Somewhat bitter and unclean, 1
3	—	—	0·20	Control, 5	Clean, 1	—	—	Good, clean, F
				Inoculated, 5	Acetic acid, 1	—	—	Somewhat bitter and unclean, 1
4	Same cheese as culture 3	Bacilli (short, thick; short chains)	0·10	Control, 6	Very good, F	—	—	Clean, mild, F
				Inoculated, 6	Sweet, 1	—	—	Somewhat bitter and unclean, 1
4	—	—	0·20	Control, 7	Very good, F	—	—	Slightly tart, F
				Inoculated, 7	A little peculiar, 1	—	—	Bitter, unclean, 2
5	Poor quality immature cheese	Coccobacillary forms (small; fairly long chains.) (On agar bacilli, small; short chains)	0·10	Control, 8	Clean, good, F	—	—	Sharp, acid, 1
				Inoculated, 8	Sweet, 1	—	—	Fermented, 2
5	—	—	0·20	Control, 9	Clean, good, F	—	—	Full, mature, 1
				Inoculated, 9	Sweet, 1	—	—	Unclean, fermented, 2
6	Poor quality immature cheese (raw milk)	Coccobacillary forms (small; short chains) (on agar bacilli, small; short chains)	—	Control, 10	Very good, F	—	—	Inclined bitter, 1
				Inoculated, 10	Good, 1	—	—	Mature, better than control, 1
6	—	—	0·20	Control, 11	Clean, good, F	—	—	Good, little lacking in bouquet, 1
				Inoculated, 11	Clean, good, F	—	—	Good, little lacking in bouquet, 1
6	—	—	0·20	Control, 12	Clean, good, F	—	—	Strong, bitter, saline, 1
				Inoculated, 12	Good, 1	—	—	Mature, better than control, 1
7	Same cheese as culture 6	Coccobacillary forms (small; short chains) (on agar bacilli, small; short chains)	0·20	Control, 13	Good, clean, F	—	—	Saline, little metallic, 1
				Inoculated, 13	Good, 1	—	—	Bitter, worse than control, 1

* Unfortunately, repetition with this culture was prevented through the fall in milk supply at the end of the season.

Table II (*continued*)

F = finest, 1 = 1st grade, 2 = 2nd grade

Cultures employed				Cheeses resulting				
					Remarks on flavour and the grade of cheese at different ages			
Culture no.	Source of culture	Appearance of organisms in milk culture	% inoculum	Cheese no.	2 weeks	4 weeks	6 weeks	17 weeks
7	—	—	0·20	Control, 14	Good, clean, F	—	—	Mature, 1
				Inoculated, 14	Good, 1	—	—	Sharp, bitter, worse than control, 1
7	—	—	0·20	Control, 15	Clean, flat, 1	—	—	Bitter, 2
				Inoculated, 15	Very good, acid, F	—	—	Sharp, acid, bitter, 2
8	Good quality mature cheese	Bacilli (short, thick; short chains)	0·01	Control, 16	—	Normal, 1	Mild, 1	Mild, 1
				Inoculated, 16	—	Slight "diacetyl" flavour, 1	Mild, little better than control, 1	Similar to control, 1
8	—	—	0·02	Control, 17	—	Slightly unclean, 1	Unclean, fermented, 2	Off, "Burnt rubber", 2
				Inoculated, 17	—	Better than control, 1	Clean, full acid, 1	Mild, mature, 1
9	Poor quality mature cheese	Bacilli (short, thick; short chains)	0·02	Control, 18	—	Very good, F	Mild, 1	Good, mild Cheddar, F
				Inoculated, 18	—	Very strong, fermented, fishy, 2	Very strong, fermented, 2	Strong, pungent, fermented, 2
9	—	—	0·05	Control, 19	—	Fair, 1	Clean, 1	Mild Cheddar, 1
				Inoculated, 19	—	Strong, fermented, 2	Strong, fermented, 2	Strong, pungent, fermented, 2
10	Very good quality mature cheese (organisms isolated by enrichment)	Bacilli (short, thick; short chains)	0·02	Control, 20	—	Fair, 1	Clean, mild, F	Good, clean, F
				Inoculated, 20	—	Sweet, fermented, 2	Winey, fermented, 2	Strong, yeasty, fermented, 2
10	—	—	0·20	Control, 21	Clean, good, F	—	—	Sharp, mature, 1
				Inoculated, 21	Fermented, 1	—	—	Strong, fermented, 2
10	—	—	0·20	Control, 22	Clean, 1	—	—	Fair, 1
				Inoculated, 22	Very sharp, acid, 2	—	—	Strong, fermented, 2
10	—	—	0·20	Control, 23	Clean, 1	—	—	Fair, 1
				Inoculated, 23	Fermented, 2	—	—	Strong, fermented, 2
11	Very good quality immature cheese	Bacilli (thin; short chains)	0·02	Control, 24	—	Clean, 1	Trace, unclean, F	Very good, shade strong, F
				Inoculated, 24	—	Sharp, but full, F	Fair, 1	Strong, shade bitter, little worse than control, 1
11	—	—	0·10	Control, 25	—	Very good, F	Very good, F	Sharp, 1
				Inoculated, 25	—	Very good, F	Shade bitter, F	Sharp, 1
11	—	—	0·20	Control, 26	—	Slightly unclean, 1	Good, clean, acid, F	Mild, mature, 1
				Inoculated, 26	—	Inclined, sweet, 1	Not as good as control, 1	Strong, inclined fermented, 1
11	—	—	0·20	Control, 27	—	Slightly bitter, 1	Good, 1	Clean, but a little insipid, 1
				Inoculated, 27	—	Shade cleaner than control, 1	A little acid, 1	Bitter, worse than control, 1

Table II (*continued*)

F = finest, 1 = 1st grade, 2 = 2nd grade

Cultures employed				Cheeses resulting				
					Remarks on flavour and the grade of cheese at different ages			
Culture no.	Source of culture	Appearance of organisms in milk culture	% inoculum	Cheese no.	2 weeks	4 weeks	6 weeks	17 weeks
12	Good quality mature cheese	Bacilli (thick; short chains)	0·10	Control, 28	—	Very good, F	Good, clean, F	Normal, 1
				Inoculated, 28	—	Similar to control, F	Similar to control, F	A little sharp, 1
12	—	—	0·05	Control, 29	—	Very good, F	Insipid, 1	Clean, 1
				Inoculated, 29	—	Similar to control, F	Shade bitter, F	A little bitter, 1
12	—	—	0·02	Control, 30	—	Normal, 1	Slightly sharp, 1	Clean, mild, 1
				Inoculated, 30	—	Slightly saline,1	Pleasant, 1	Slightly saline, 1
12	—	—	0·01	Control, 31	—	Normal, 1	Normal, 1	Clean, mild, 1
				Inoculated, 31	—	Slightly better than control, 1	Sharper than control, 1	A little more attractive than control, 1
11	—	—	0·10	Control, 32	—	Fair, 1	Good, clean, F	Clean, slightly acid, 1
+	—	—	+	Inoculated, 32	—	Fair, 1	Slightly better than control, F	Mild, "diacetyl", better than control, F
12			0·10					
11	—	—	0·05	Control, 33	—	Fair, 1	Good, clean, F	Good, clean, F
+	—	—	+	Inoculated, 33	—	Aromatic, slightly better than control, 1	Very good, "diacetyl", F	Good, clean, F
12			0·05					
11	—	—	0·10	Control, 34	—	Clean, neutral, 1	Good, 1	Clean, little insipid, 1
+	—	—	+	Inoculated, 34	—	Cleaner than control, more cheese flavour, 1	Clean, mature, better than control, F	More cheese flavour than control, 1
12			0·05					
13 M	Good quality mature cheese (mixture of 10 pure cultures)	Bacilli	0·10	Control, 35	—	Not quite clean, 1	Very good, F	Clean, good, F
				Inoculated, 35	—	More cheese flavour than control, 1	Very good, F	Mild, mature, "diacetyl", F
13 M	—	—	0·10	Control, 36	—	Fair, 1	Clean, 1	Good, trace, "diacetyl", 1
				Inoculated, 36	—	Slightly saline, 1	Good, 1	Attractive, "diacetyl", 1
14 M	Good quality English cheese (10 pure cultures)	Cocci (chains)	0·15	Control, 37	—	Clean, neutral, 1	Good, 1	Clean, but little insipid, 1
				Inoculated, 37	—	Clean, more flavour than control, 1	Clean, mature, better than control, F	Slightly better than control, 1
14 M	—	—	0·15	Control, 38	—	Sharp, acid, 1	Clean, acid, 1	Clean, mild, 1
				Inoculated, 38	—	Slightly cleaner than control, 1	Sharp, acid, worse than control, 1	Similar to control, 1
14 M	—	—	0·15	Control, 39	—	Normal, 1	A little flat, 1	Slightly bitter, 1
+			+	Inoculated, 39	—	Sharp, acid, 1	Mild Cheddar, "diacetyl", F	Better than control, 1
11			0·05					
14 M	—	—	0·15	Control, 40	—	Clean, acid, 1	Full, acid, 1	Mild, clean, 1
+			+	Inoculated, 40	—	Sharp, acid, 1	Better than control, 1	Fuller flavour than control, F
12			0·10					

Volatile acid formation.

0·5 ml. clotted culture (in yeast-milk) was added to 150 ml. autoclaved milk containing 5 per cent of yeast extract. After an incubation period of 7 days at 31° C. the culture was diluted with an equal volume of water, 7 ml. $2N$ H_2SO_4 added and the mixture was steam-distilled at such a rate that 1000 ml. distillate collected in 80 min. In the preceding table the figures represent the number of ml. $N/10$ NaOH required to neutralize the 1000 ml. distillate minus the number of ml. $N/10$ NaOH required for 1000 ml. distillate from a sterile milk "blank".

Carbon dioxide evolved in yeast-milk culture.

The method employed was purely comparative. The organisms were sown into 50 ml. autoclaved yeast milk (10 per cent) contained in 100 ml. Erlenmeyer flasks. The carbon dioxide evolved, during a period of 4 days, passed through a sterile connecting tube loosely plugged with cotton-wool and was absorbed in $N/10$ $Ba(OH)_2$ in another flask.

It will be seen from the two preceding tables that cultures 5, 6, 7, 9 and 10 all formed gas rapidly, did not thrive in milk or reduce litmus in this medium, and failed to ferment mannitol and salicin. It would seem, therefore, that these five strains should be classed as Betabacteria (Orla-Jensen).

The remaining seven strains of lactobacilli were, presumably, Streptobacteria. They did not form carbon dioxide to any large extent, clotted milk fairly rapidly, and usually brought about reduction in litmus milk (exceptions —cultures 4, 8 and 11). All fermented maltose and salicin, and, with the exception of cultures 3 and 4, formed acid from mannitol.

Although the largest amounts of volatile acid (in yeast-milk culture) were produced by certain strains of Betabacteria, other organisms in this same group (e.g. cultures 5 and 10) formed less volatile acid than did many of the Streptobacteria examined. It is interesting to note that the most heat-resistant strains (nos. 5, 6 and 7) among the twelve cultures were Betabacteria which assumed cocco-bacillary forms in milk.

Discussion

It has been seen from the tables that inoculation of the cheese-milk with various strains of lactobacilli caused the development of specifically characteristic flavours in the cheese. These flavours were of many types, both bad and good depending on the strain of bacteria added. It is worthy of mention that when the inoculated cheeses were examined at an age of 14 days, they frequently possessed an unusually acid taste which, however, was only transient and probably arose from the early proliferation of lactobacilli.

It was noted, under series I, that the addition to the cheese-milk of natural mixed cultures from mature cheese of either good quality or poor quality led to the production of unclean, fermented flavours in the resultant cheese. It is clear, from cheeses 18–23 in series II, that identical flavours were

Table III

Incubation temperature 31° C

Culture no.	No. o days to clot milk containing 10% of yeast extract	No. of days to clot milk	Reaction with litmus milk containing 10% of yeast extract	Reaction with litmus milk	Turbidity in whey (after 2 days)	Volatile acid formation in: Yeast milk (ml. *N*/10 NaOH neutralized)	Volatile acid formation in: Yeast milk +0·4% citric acid (ml. *N*/10 NaOH neutralized)	Formation of nitrite (Pake's nitrate medium)	CO_2 evolved in yeast-milk culture (ml. *N*/10 $Ba(OH)_2$)
1	3	5	Rapid: acid, clotting, reduction	Acid, clotting, reduction	Marked	5·5	6·5	Nil	4·7
2	3	10	Rapid: acid, clotting, reduction	Acid, clotting, reduction	Nil	18·0	31·5	Nil	2·9
3	2	8	Rapid: acid, clotting, reduction	Acid, clotting, reduction	Nil	8·6	10·0	Slight	4·7
4	2	6	Rapid: acid, clotting, reduction	Acid, clotting	Nil	8·0	7·6	Slight	4·9
5	3	10	Rapid: acid, clotting, reduction	Acid, clotting	Marked	0·0	0·5	Nil	12·3
6	4	14	Acid, clotting, reduction	Acid	Nil	20·1	36·4	Nil	14·1
7	4	14	Acid, clotting reduction	Acid	Nil	18·1	33·3	Nil	20·8
8	3	12	Acid, clotting	Acid	Marked	17·0	17·0	Nil	2·9
9	14	>30	A little acid	A little acid	Nil	22·5	45·5	Nil	10·1
10	14	>30	A little acid	A little acid	Nil	9·0	7·0	Nil	11·0
11	3	9	Acid, clotting, reduction	Acid, clotting	Slight	17·5	25·0	Nil	3·7
12	2	9	Acid, clotting, reduction	Acid, clotting, reduction	Slight	18·0	17·4	Nil	3·7

Culture no. 1 was distinctive in that it formed extremely small colonies on agar media. Cultures 3 and 4, when first isolated, displayed a tendency to reduce nitrate; this power was lost on repeated subculturing in yeast-milk.

Cultures nos. 5, 6 and 7 were somewhat more heat resistant than the remainder of the cultures, the thermal death-point for these three strains being slightly higher than 140° F./½ hour.

Table IV. *Sugar reactions*

Culture no.	Arabinose	Dextrose	Mannitol	Sucrose	Lactose	Maltose	Salicin	Inulin	Starch
1	+	++	++	+	++	++	++	−	−
2	++++	++++	++	++	++++	++++	++++	−	−
3	+	++	−	++	++	++	++	−	−
4	+	++	−	++	++	++	++	−	−
5	−	−	−	++	−	−	−	−	−
6	−	−	−	++	++	++	−	−	−
7	−	±	−	+	+	+	−	−	−
8	+	+++	++	+++	++++	+++	+++	−	−
9	+++	++	+	+	+++	++++	−	−	−
10	+++	++	+	+	+++	++++	+	−	−
11	−	++	++	++	++++	+++++	++++	−	−
12	+	++++	+++	++++	++++	+++++	+++	−	−

† A casein digest medium (total nitrogen =0·5 per cent) containing 2 per cent of the sugar was employed. The cultures were incubated at 31° C. for 14 days.

In the table − represents an increase of less than 0·2 per cent lactic acid. + represents an increase of 0·2 per cent lactic acid. ++ represents an increase of 0·4 per cent lactic acid, and so on.

produced by certain pure cultures of lactobacilli (e.g. nos. 9 and 10). The first strains which were found to be capable of imparting these flavours were isolated from a poor quality mature cheese. More recently, similar strains have been isolated in large numbers from samples of green cheese, 14 days old, obtained from the Wellington Dairy Produce Grading Stores. The flavours of these cheeses were described by the Dairy Division graders as "fermented".

During the course of early experiments, attempts were made to culture lactic bacteria in sterilized cheese. Portions of green cheese, autoclaved at 12–15 lb., were sown while still warm, with many strains of lactobacilli and cocci. It was found that whereas starter streptococci did not flourish in this medium, lactobacilli usually grew very well. Strains of lactobacilli which formed acid very slowly in milk (e.g. culture 9) remained viable in sterilized cheese much longer than strains capable of more rapid acid production.

The discovery was then made, that by inoculating autoclaved cheese with *natural* mixed cultures of lactobacilli from mature or nearly mature cheeses, whether of good quality or poor quality, it was invariably possible to isolate from the autoclaved cheese cultures, after an incubation period of approximately 2 months, resistant strains of lactobacilli which appeared to be of the same type as culture 9. Examination of the original cheese by direct plating methods usually failed to reveal the presence of these organisms. It would seem that these strains occurred in all of the normally manufactured mature cheeses that were studied; apparently, the organisms were generally present in very small numbers which did not multiply to any marked extent until, with increasing cheese age, many of the other types present died off. Whether these organisms are of widespread occurrence in New Zealand cheese, as appears probable, will be revealed by future work. Repeated transfers in autoclaved cheese of a natural mixed cheese culture did not, after the first transfer, raise the proportion of these strains present. The most salient characteristic of the organisms, which appear to be Betabacteria, lies in the slowness with which they clot yeast-milk (2–3 weeks at 31° C.); ordinary steamed milk, of course, is clotted much more slowly.

It seems that the yeast-milk cultures of the natural mixtures of lactobacilli which were employed in series I contained sufficient numbers of these organisms to cause the development of fermented flavours in the inoculated cheeses. As would be expected, inoculum 3 N, from an immature good quality cheese, apparently contained such a very small number of bacilli of culture 9 type (none could be isolated by culturing in autoclaved cheese) that the inoculated cheese did not possess a fermented flavour at maturity, although such a flavour threatened to develop at an earlier age.

Cheeses 16 and 17 illustrate an interesting point of practical importance which was frequently observed during the course of this investigation. Since the quality of the milk usually employed was high, the inoculation of culture 8 did not result in inoculated cheese 16 being any better than the control cheese. However, on the day when cheeses 17 were manufactured, the milk

before pasteurization was found to be "slightly unclean". As will be seen, control cheese 17 developed during maturation an "off-flavour", which evidently arose from the presence of undesirable types of flavour-forming organisms derived, in all probability, from the milk. Now, the addition of culture 8 to this milk resulted in the development of a clean desirable flavour in inoculated cheese 17. Apparently, proliferation in this cheese of the harmful types of bacteria from the original milk was suppressed by the presence of the large number of desirable lactobacilli from culture 8.

The possibility of suppressing the growth of objectionable organisms during cheese ripening, on a commercial basis, by "swamping" in this way with lactobacilli of a desirable strain appears quite attractive. The production of clean milk and the use of a good starter eliminates the chance of cheese spoilage through the development of non-lactic bacteria, but it does not ensure the presence of the best flavour-forming lactic organisms. It is conceivable that, as the quality of the milk supply is improved, i.e. the number of bacteria is reduced, the small number of lactobacilli present may not include strains which are capable of developing desirable flavours during cheese maturation, and it may prove necessary to add such strains in order to produce the best cheese.

The complexity of flavour formation in cheese is well illustrated by the results obtained in the later experiments. It will be seen that the addition to the cheese-milk of either culture 11 or culture 12, singly, did not improve the flavours of the resultant cheeses; in fact, the flavours of the control cheeses were preferred. However, addition of cultures 11 and 12 in admixture yielded a pleasant aromatic flavour, presumably through symbiosis or through the "compounding" of two separate flavours. A similar effect was obtained with culture 13 M, which was a mixture of ten pure strains. The use of culture 14 M did not bring about any appreciable differentiation between control and experimental cheeses, but when a small proportion of either culture 11 or 12 was added, the experimental cheeses were slightly preferred to the controls.

It has been seen from Tables III and IV that the lactobacilli which formed the most objectionable flavours in cheese should probably be classed as Betabacteria, while the more desirable flavour-forming organisms usually appeared to be strains of Streptobacteria (no attempt has been made here to distinguish between *Sbm. casei* and *Sbm. plantarum*).

Even among the limited number of strains of lactobacilli (12) examined in this investigation, however, exceptions were found, viz. culture 6 which, although a Betabacterium, produced a desirable cheese flavour, and cultures 3 and 4 (Streptobacteria) developed unclean bitter flavours. It should also be borne in mind that the strains of Betabacteria studied were all isolated from cheese of poor or indifferent quality. If, however, subsequent work should demonstrate that certain strains of Betabacteria occur in large numbers in commercial cheese of good quality it is extremely probable that such strains will also prove to have desirable flavour-forming properties.

In the course of the experiments described in this communication, various strains of streptococci, which were found to constitute a large proportion of the flora of occasional mature cheeses, were inoculated into cheese-milk. Only one set of cheeses prepared with the addition of cocci is cited here (Table II, cheeses 37 and 38), for the reason that these experiments have not yet been completed. There is considerable evidence, however, that certain slow acid forming strains of streptococci which occur relatively infrequently in cheese are capable of modifying cheese flavour in much the same way as do lactobacilli.

From a general consideration of the experimental results it would appear that the group of lactobacilli (and to a lesser extent certain resistant strains of cocci) are of extreme importance in governing cheese flavour. The range of characteristic flavours developed in the cheeses examined comprised types commonly encountered in commerce—being described as "aromatic", "diacetyl-like", "bitter", "unclean", "fermented" and "metallic", and it seems entirely probable that such flavours, in general, arise not only from varying manufacturing conditions (the importance of this factor has long been recognized) but also from the presence of lactic organisms similar to those described in this communication. The results of the above experiments indicate quite clearly that deliberate modification of cheese flavour on a commercial basis by addition of selected types of lactobacilli is a practicable operation. Many difficulties in this regard, however, are apparent. One of the greatest, probably, lies in the factor of personal preference for different shades of flavour. During the course of the present investigation it was found that graders often expressed significantly different preferences and further sometimes actually acquired a liking for certain characteristic flavours which, on first acquaintance, failed to impress favourably.

It is hoped, in projected work upon cheese flavour, to study the effect of varying between greater limits the amount of inoculum and the influence of symbiotic phenomena. Attention will be given also to the possibility that, in order to obtain the best results from the addition of various organisms, modification of working conditions to suit the particular strains of lactobacilli employed may be necessary.

In any case, the desirability of adding strains of lactobacilli to the cheese vat on a large scale depends on the selection of organisms which do not introduce cheese defects such as openness and discoloration. The influence of lactobacilli upon the development of these defects will be discussed in later communications.

Summary

Various strains of lactobacilli isolated from Cheddar cheese were added to the milk employed in cheese manufacture. Different strains of these organisms were found to impart specifically characteristic flavours to the cheese. The range of flavours developed included both desirable and objectionable types,

many of which are commonly encountered in commercial cheese, e.g. "pleasant aromatic", "diacetyl-like", "metallic", "bitter", "unclean" and "fermented".

The inoculation of cheese-milk with natural mixtures of lactobacilli from mature cheeses led almost always to the development of unclean "fermented" flavours. The causative strains of lactobacilli were isolated and shown to be of frequent occurrence in normal mature cheeses but under ordinary circumstances the actual numbers of these bacilli present in most cheeses were too small to spoil the flavour.

The results of these experiments were taken to indicate that the group of lactobacilli constitute the most important flavour-forming factor in normal Cheddar cheese.

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161. STUDIES IN CHEDDAR CHEESE

VI. THE DEGRADATION OF MILK PROTEINS BY LACTIC ACID BACTERIA ISOLATED FROM CHEESE, ALONE, WITH STERILE RENNET AND WITH WHOLE RENNET

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Introduction

The exact role of the enzymes of rennet, of milk and of the lactic acid bacteria in cheese-ripening (considered from the point of view of protein degradation) has been a subject of much controversy. Exact knowledge of the significance of each "factor" can only be obtained by the elimination of all other factors. Unfortunately the conditions controlling cheese-ripening are so complex that clear-cut experiments are very difficult. Thus the proteolytic enzymes of rennet and the lactic acid bacteria have not, as far as we know, been studied under the conditions in which they would work in cheese. Experiments on these enzymes have usually been made, with separated milk as a substrate, under conditions differing from those in ripening cheese. However, the results obtained from such experiments may serve as a guide to what may be expected to occur in cheese itself.

Cheddar cheese of typical quality made from low count milk (plate count of about 1000 per ml. and *coli* absent in 1 ml.) have been shown by Allen (1) and Davies *et al.* (2) to contain about 30 per cent of their nitrogen as non-protein nitrogen after about 5 months' ripening at a temperature of about 14° C. Such cheese possess a flora that is practically entirely true lactic acid bacteria (3). It is generally assumed that the proteolysis must be due to the combined action of the enzymes of these bacteria, and of rennet. The original bacteria of the milk and rennet are so overgrown by the rapidly growing streptococci and further inhibited by the acidity (*p*H 5·0–5·5) and low potentials produced (4) that they do not thrive in the cheese. It is only under conditions that exist in very sweet cheese (*p*H above 6·0) that these organisms are able to grow and then produce, in some cases, taints such as "stinker cheese" (5, 6).

In this paper are recorded the results of investigations into the proteolytic activities of pure cultures of lactic acid bacteria isolated from normal Cheddar cheese made from clean milk. Experiments have been made with the additions of rennet sterilized by filtration through a Seitz filter, of whole rennet with its natural flora, and of chalk.

Experimental methods

The cultures used have been described in a previous paper(3). They were inoculated (from milk cultures) into 30 ml. quantities of separated milk (sterilized by three steamings at 100° C. for half an hour and tested for sterility) contained in boiling tubes. Chalk, when added, was sterilized before and after incorporation into the medium. The filtered rennet was tested for sterility by plating on suitable media.

After inoculation the tubes were sealed with paraffin wax above the plug and incubated for 24 hours at 37° C. and then at 15° C. for the duration of the experiment, these conditions being approximately those used in cheese manufacture.

Analytical procedure

A microscopic test was first made for purity of the culture and the whole medium then thoroughly mixed. Portions were weighed out and the following determinations made in duplicate:

(1) *Total nitrogen* by the Kjeldahl method.

(2) *Protein nitrogen* by precipitation with 4 per cent trichloracetic acid.

(3) *Total amino (Sörensen) nitrogen* by a second titration with sodium hydroxide after the addition of neutralized formalin and alcohol (to make a final concentration of 50 per cent alcohol) to the end-point of phenolphthalein.

(4) *Non-protein Sörensen nitrogen* by a similar titration on the trichloracetic acid filtrate.

(5) *Titratable acidity.* This value was obtained by the first titration in the total amino (Sörensen) nitrogen titration.

(6) *Hydrogen-ion concentration.* This was measured with the quinhydrone electrode, standardized by a standard phthalate buffer at *p*H 3·97(7, 8).

The results recorded are the means of satisfactory duplicates; when a satisfactory agreement was not obtained, the result has been omitted. The difficulties experienced in this matter were due to the errors of sampling, which tend to be large in a semi-solid medium, and were chiefly found in the experiments with chalk.

The accuracy of the determinations was approximately as follows: non-protein nitrogen ±0·5 per cent, total Sörensen nitrogen ±2 per cent, non-protein Sörensen nitrogen ±1 per cent (expressed as percentage of total nitrogen), acidity (as lactic acid) ±0·05 per cent of the culture, hydrogen-ion concentration ±0·02 *p*H unit.

First series

In this series cultures were made

(1) in separated milk alone;

(2) in separated milk plus filtered sterile rennet (0·1 ml. of 10 per cent rennet);

(3) in separated milk plus chalk;

(4) in separated milk plus chalk and filtered sterile rennet (0·1 ml. of 10 per cent rennet).

The age of the cultures at the time of analysis was from 60 to 68 days. The results are given in Table I.

Discussion.

The increases in protein degradation products in the 60 days holding period at 15° C. are small and vary considerably from strain to strain. Without the rennet enzymes the greatest increase in non-protein nitrogen is only about 2 per cent (7·8–10); when the rennet is also present, however, increases of about 12 per cent (7·8–20) are found. Chalk alone has no effect on the non-protein nitrogen. This does not prove that the reaction of the medium is of no importance, however, since the use of chalk as a neutralizing agent is extremely unsatisfactory unless the culture can be continually shaken. This was impossible in the present experiment with clotted milk in tubes. Kelly (9) has shown that, after shaking such tubes, the *p*H of the clotted milk may change, for example, only from 4·78 to 5·14, milks of higher initial *p*H values being still less affected. Manometric experiments have shown that chalk will react with a phosphate buffer having a *p*H as high as 6·6 (10). The efficacy of chalk as a neutralizing agent, therefore, is entirely a matter of agitation and velocity of reaction. Increases in the Sörensen nitrogen figures are similarly not very marked either in the case of the cultures alone, or with the rennet. With the lactobacilli the addition of chalk resulted in an increase in the amount of free amino nitrogen.

The results are generally in accord with the accepted view that proteolysis by lactic acid bacteria is a function of endogenous enzymes liberated on autolysis of the cell. The increases are small, gradual and somewhat irregular, as might be expected, since a period of incubation of 60 days at 15° C. would not necessarily result in complete autolysis. The results are in very good agreement with those of Kelly (9).

Second series

In these experiments cultures were made (1) alone, (2) with rennet sterilized by filtration and (3) with whole rennet. A more satisfactory agreement between duplicate results was obtained in this series, chalk not being used. The age of the cultures was from 101 to 104 days. The results are given in Table II.

Discussion.

Considerably higher values for the "culture plus rennet enzymes" tubes were found and are perhaps due to the longer time of the experiment (100 as compared with 60 days). An average value of about 30 per cent of non-protein nitrogen was obtained. This value is of the same order as that obtained

Table I. *Protein degradation by lactic acid bacteria with sterile rennet and with chalk*

Experimental conditions	% of total nitrogen: Non-protein N		Sörensen N		Non-protein Sörensen N		Final acidity as % lactic acid		Final *p*H	
Without chalk:										
Separated milk alone (uninoculated control)	7·8	10·0	6·9	7·4	3·8	4·3	0·21	0·18	—	—
,, +*S. lactis* (Orla Jensen)	8·8	15·6	6·0	6·6	3·3	2·3	0·88	0·94	4·51	4·43
,, +*S. cremoris* (Orla Jensen)	7·0	11·4	6·8	6·1	1·6	3·7	0·76	0·77	4·76	4·76
,, +*S. cremoris* 3·4	—	16·5	6·1	5·8	3·0	3·2	0·83	0·85	4·52	4·54
,, +*S. cremoris* 3·12	10·0	13·0	7·4	6·5	3·8	3·5	0·81	0·80	4·50	4·48
,, +*Sbm. plantarum* 1·4	8·3	6·2	6·3	6·5	2·7	3·4	0·84	0·80	—	—
,, +*Sbm. plantarum* 1·8	8·2	10·3	6·9	7·3	2·1	3·7	0·57	0·72	—	—
,, +*Sbm. casei* 2·9 (intermediate)	8·5	13·3	6·7	7·0	3·3	3·9	0·62	0·77	4·86	4·61
,, +*Sbm. casei* 2·13	7·7	20·0	9·7	8·8	3·7	5·9	1·12	1·19	4·26	4·02
,, +*Sbm. casei* 2·24	7·7	7·2	6·8	9·0	2·5	4·7	0·44	0·38	—	—
With chalk:										
Separated milk alone (uninoculated control)	7·5	10·5	6·3	6·4	2·4	2·8	0·13	0·13	6·78	6·71
,, +*S. lactis* (Orla Jensen)	6·7	8·7	6·9	6·6	1·8	2·3	0·74	0·76	4·74	4·86
,, +*S. cremoris* (Orla Jensen)	—	17·0	7·6	8·1	—	3·3	0·64	0·69	4·72	4·73
,, +*S. cremoris* 3·4	—	9·8	7·7	7·2	3·2	2·5	0·82	0·80	4·85	4·78
,, +*S. cremoris* 3·12	6·5	12·5	7·6	6·2	2·9	2·4	0·81	0·80	4·95	4·90
,, +*Sbm. plantarum* 1·4	—	14·2	16·6	18·9	4·5	3·6	0·96	1·14	—	—
,, +*Sbm. plantarum* 1·8	—	11·7	13·6	14·5	2·6	2·2	0·94	1·32	—	—
,, +*Sbm. casei* 2·9 (intermediate)	—	17·5	10·4	5·5	3·7	3·8	0·74	0·79	5·77	4·73
,, +*Sbm. casei* 2·13	7·5	13·0	5·7	6·0	3·4	5·7	0·82	1·00	4·81	4·37

The first column gives the results without, the second column with, filtered sterile rennet.

Table II. *Protein degradation by lactic acid bacteria alone, with sterile rennet and with whole rennet*

Experimental conditions	Expressed as % of total nitrogen: Non-protein N			Sörensen N			Non-protein Sörensen N			Final acidity as % lactic acid			Final *p*H		
Separated milk alone (uninoculated control)	5·0	10·0	71·0	3·1	4·2	13·0	0·0	0·9	11·4	0·19	0·21	0·63	6·48	6·35	5·31
,, +lactic acid solution	9·0	16·0	63·0	3·1	4·3	5·8	0·0	1·3	4·2	1·18	1·09	1·38	3·82	4·01	3·83
,, +*S. lactis* (Orla Jensen)	6·4	33·6	74·4	—	—	—	0·0	1·6	6·1	0·94	0·96	1·16	4·30	4·30	4·13
,, +*S. cremoris* (Orla Jensen)	10·6	37·8	76·4	4·2	4·9	8·1	0·5	2·0	6·5	0·81	0·82	1·05	4·68	4·66	4·24
,, +*S. cremoris* (3·4)	4·0	26·0	74·0	2·5	3·2	4·4	0·0	1·2	4·8	0·88	0·88	1·02	4·48	4·47	4·14
,, +*Sbm. plantarum* 1·4	1·5	35·0	80·0	1·9	3·5	8·2	0·5	2·2	8·2	0·90	1·18	1·43	4·58	4·28	3·98
,, +*Sbm. plantarum* 1·8	3·6	36·0	79·0	4·0	5·1	12·0	0·6	2·9	11·6	0·69	0·82	1·13	—	—	—
,, +*Sbm. casei* (intermediate) 2·9	4·0	32·0	80·0	2·0	4·9	8·3	0·8	3·4	9·7	0·82	1·10	1·55	—	4·19	3·82
,, +*Sbm. casei* 2·13	2·0	30·0	71·0	4·5	5·4	8·5	0·0	2·2	7·8	1·27	1·28	1·52	4·07	4·06	3·85
,, +*Sbm. casei* 2·24 (weakened)	2·0	24·0	96·0*	—	4·9	39·2*	1·0	1·5	38·4*	0·28	0·79	0·54*	6·00	—	5·89*

The first column gives the results without rennet, the second column with filtered sterile rennet and the third column with whole rennet.

* Culture 2·24 was a weakened strain of *Sbm. casei* (see Ref. (3)) and was overgrown by the organisms in the rennet. It is of interest to note that in all other cases the lactic acid bacteria outgrew the rennet organisms as indicated by the final *p*H of the culture.

by Allen (1) and Davies *et al.* (2) for clean milk Cheddar cheese. It appears likely, therefore, that in the combined activities of the rennet enzymes and the true lactic acid bacteria are to be found all the factors responsible for the ripening of the cheese as far as the non-protein nitrogen is concerned. The Sörensen values, however, show no significant increase. This may be due to the fact that the peptidase of rennet is markedly inhibited by an acid reaction whereas the proteinase is not, i.e. the proteinase (*papainase*) is readily active at *p*H 4–6, but the peptidase, which has its optimum at *p*H about 6, is much less active at *p*H 4–5 (11). Tarnanen (12) has shown that a similar phenomenon occurs in the proteolytic enzymes of the lactic acid bacteria. Thus the proteinase of *B. casei* (*Tbm. helveticum*) has an optimum at *p*H 5·7–6·0, the polypeptidase an optimum at *p*H 6·8. It should be observed that the addition of lactic acid to produce a *p*H slightly less than that of the lactobacilli cultures does not result in the same degree of proteolysis (Table II). With whole rennet very high values were obtained for the non-protein nitrogen fractions and the final *p*H values were even lower than in the absence of rennet organisms. Marked digestion of the curd was apparent in all cases; and was found to be due to the growth of the rennet organisms; the slower the rate of growth of the lactic acid bacteria the more complete was the digestion of the casein.

General discussion of results

In vitro experiments such as those described in this paper must necessarily fail in many respects when their significance in relation to cheese-ripening is considered. These points will now be discussed:

(1) *Composition of the medium.* Milk differs markedly in composition from curd and cheese, and from the point of view of proteolysis experiments the most important differences are to be found in the sugar and water contents. An attempt was made to prepare a synthetic medium having a composition similar to that of cheese but its viscosity prevented its use.

(2) *Hydrogen-ion concentration.* Cheese (or curd) varies in *p*H from about 4·9 to 5·6; milk cultures of the lactic acid bacteria used rapidly reach a *p*H of from about 3·8 to about 4·5 and do not change further. The hydrogen-ion concentration of the milk culture is therefore about ten times that of the cheese—a very significant difference for the activity of some proteolytic enzymes, especially the peptidases.

(3) *Osmotic pressure.* Whereas the osmotic pressure of acid clotted milk is that of about 1·3 per cent NaCl, that of cheese is certainly greater than that of 5 per cent NaCl, the amount of salt added in making. This assumes complete solution of the salt in the aqueous phase and that all the water is free. It may safely be assumed that the effective osmotic pressure in the ripening cheese is considerably higher than that of 5 per cent NaCl. This must necessarily be an important factor in the cytolysis of the bacteria, and the subsequent liberation of enzymes.

(4) *Effect of heat on the medium.* Cheese is normally made from raw or pasteurized milk while *in vitro* experiments have usually been carried out in milk sterilized by heating for three periods of 30 min. at 100° C. Heating appears to favour acid production by lactic acid bacteria (13, 14); its effect on the proteolysis by lactic acid bacteria is not known, but in view of the fact that heating causes denaturation of albumin and globulin and slows down the rate of enzymic digestion of these proteins, it seems unlikely that proteolysis would proceed as rapidly in heated milk as in raw milk. In addition, Van Dam (15) has shown that the higher the temperature to which the milk is heated, the slower is the rate of ripening of the cheese.

(5) *Oxygen tension.* A certain amount of evidence has appeared that makes it seem likely that anaerobiosis may favour the proteolytic activities of the lactic acid bacteria (3, 12, 16). It is evident that the conditions will be more anaerobic in the interior of a cheese than in a boiling tube. Thus a cheese maintains an *r*H of about 3 for almost its entire period of ripening (4) while a tube culture of, for example, *Str. lactis*, will allow the litmus to reoxidize in about 3 weeks at 15° C. (litmus has an *r*H of about 7 at 50 per cent oxidation at *p*H 5).

SUMMARY

It has been shown by experiments in milk that the enzymes of commercial rennet in conjunction with the lactic acid bacteria occurring in Cheddar cheese can bring about protein breakdown similar in extent to that found in the ripe cheese as far as the non-protein nitrogen is concerned. The amino nitrogen produced is, however, much less than in cheese. This may be ascribed to the higher *p*H of cheese as compared with that of the milk cultures, since acidity adversely affects the peptidases present. Attention is drawn to the differences between the conditions.

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162. THE RHEOLOGY OF CHEESE, BUTTER AND OTHER MILK PRODUCTS

(THE MEASUREMENT OF "BODY" AND "TEXTURE")

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Rheology is defined as the science of the deformation of matter. It includes the study of all deformations, recoverable (elastic) and non-recoverable (plastic). In this paper cheese is primarily considered although the fundamental principles apply to all materials.

The elastic and plastic properties of cheese exercise a great influence in the ripening processes and the incidence of faults. Although much progress has been made in the bacteriological and chemical investigations of cheese ripening, little fundamental work on the nature of and the factors controlling the physical properties of curd and cheese has been carried out. Success in the art of cheese making lies chiefly in the production of a curd of *physical* properties that will control the resultant bacteriological and chemical changes and so produce the particular type of cheese desired (1). A knowledge of the physical structure of curd and cheese and of the factors which control it is thus of first importance in the scientific study of cheese.

Physical factors concerned in cheese ripening

Cheese making consists essentially in the clotting of milk and the separation and "drying out" of the resultant curd. The numerous varieties of cheese are due to differences in the methods used to clot the milk and dry the curd, and in the subsequent manipulations.

The basic factors which are under the control of the cheese maker are temperature, acidity and moisture—themselves interrelated. The chemical composition of the milk is another complex factor, which, although not under the immediate control of the maker, plays its part. In practice, the type of cheese made is chosen according to the locality and the milk supply. It is well recognized that the best cheese of certain makes are produced in certain districts, although no evidence can be found that this is due to differences in the chemical nature of milk secreted by cows maintained on pasture lands of different geological structure. It is difficult to obtain reliable data on the effect of soil, pasture and food stuffs on milk for cheese making on account of the numerous factors requiring careful control in such experiments.

It has been stated that silage (2) and chemical fertilizers (3) do not unfavourably affect milk for cheese making. Truninger (3) asserts that the liming of land does not increase the calcium either in the forage or in the milk. The

clotting ability of the milk is affected however, and this is more dependent on the method of combination of calcium than on its absolute amount. Heltstrand (4) found that good cheese could not be produced if the cows were fed on large quantities of succulent feeds but that good hay produced milk giving good cheese. He also found that the feeding of hay to a cow giving milk that failed to clot with rennet resulted in a gradual improvement in the clotting power of the milk, which ultimately became normal. The suitability of a milk for cheese making is largely determined by the ease with which it clots with rennet. The general behaviour of the curd during ripening is, however, quite as important from the point of view of the production of good cheese. Hitherto no standard method for the investigation of curd characteristics during ripening has been available. Such a method is essential for the study of the relation between the chemical nature of the milk and the quality of the cheese, butter or any other milk product. However, one or two fundamental facts may be accepted unreservedly.

Thus it is well established that calcium is an important factor in controlling the "body" (firmness or consistency) of cheese (5). Acidity and moisture, especially the "bound" to "free" water ratio, are also important. Similarly, texture is of fundamental importance in the ripening of Stilton cheese, the development of internal cracks at the appropriate stage leading to growth of *Penicillium roqueforti* inside the cheese and the development of the true Stilton flavour (6).

Cheese grading—"body" and "texture"

So much importance is attached to the rheological properties of cheese that in England about 40 out of 100 marks awarded in grading are given for "body" and "texture". Although some authorities do not distinguish clearly between these terms it is preferable to define "body" as that quality which is perceptible to the touch; "texture" as that which is evident to the eye, excluding colour. Under the term "body" are included several factors, of which firmness (viscosity and modulus), springiness (elasticity [1]) and smoothness (homogeneity) are most important. In grading, firmness is indicated as (1) very soft, (2) soft, (3) rather soft, (4) normal, (5) firm, or (6) over-firm, and smoothness as (1) silky, (2) chalky, or (3) gritty. Texture varies in meaning in different localities, but is frequently taken to include both closeness (absence of cracks) and shortness or brittleness (easy breaking of a plug).

It is evident that methods for the assessment of the rheological properties of cheese are at present very inadequate. Our knowledge of the factors controlling such properties as "body" and "texture" is also meagre, and the first step in the study of these factors must be a satisfactory method of following quantitatively the changes occurring in different types of cheese during ripening.

From time to time special methods have been described for measuring the "spreadability" of butter and similar properties of other milk products. Such methods, however, involve special and often complicated apparatus

[1] See p. 255.

which may be difficult to standardize and may involve special units incapable of expression in centimetre-gramme-second (C.G.S.) units.

The technique described in this paper requires only the simplest apparatus and is capable of giving results in C.G.S. units. Reproducibility in different laboratories is thus made much easier.

Rheological studies of other biological substances have been made with profit in many instances. As examples may be mentioned the work of Keen & Scott Blair on soils (7), of Schofield, Halton & Scott Blair on dough (8) and of Scott Blair & Morland on honey (9).

THE PHYSICAL NATURE OF CHEESE

Cheese is a typical example of that state of matter which exhibits properties intermediate between those of a solid and those of a fluid and to which the term *plastic* may conveniently be given. In this paper the following typical properties of a plastic body will be considered: (1) *elasticity*[1] (shear modulus), (2) *viscosity*, (3) *time of relaxation* (springiness), (4) *internal viscosity*, (5) *structural viscosity*, (6) *work hardening*, (7) *true hysteresis* and (8) *relaxation flow*. Many of these properties are interrelated.

The appropriate definitions and formulae are given in Tables I and II.

Table I. *Definitions*

m = mass in g. placed on material
a = area of cheese plug
L = original length of plug
mg = weight
mg/a = stress (tensile) = S
$mg/3a$ = shear stress = S' (as Poisson's ratio is approximately 0·5)
e = elastic (recoverable) deformation
f = absolute flow (non-recoverable deformation)
t = time during which load allowed to act

e/L = elastic or recoverable strain
f/L = plastic or non-recoverable strain
} when strains are small, e.g. e and f are $<10\%$ L, otherwise $\log_e[L/(L+e)]$ and $\log_e[L/(L+f)]$ must be used

E = Young's modulus
n = shear modulus = $E/3$ — $\log n = pM$
η = viscosity — $\log \eta = pV$
t_r = time of relaxation — $\log t_r = pS$
V_s = structural viscosity
t' = time required for elastic recovery
η_i = internal viscosity (responsible for elastic after-effect)
WH = work hardening
H_T = true hysteresis
f_r = flow during relaxation (if any)

Table II. *Formulae (defining plastic behaviour)*

Young's modulus:

$$E = \frac{\text{loading stress}}{\text{recoverable strain}} = \frac{\text{mass} \times 981}{\text{area} \times \dfrac{\text{elastic stretch}}{\text{original length}}}$$

$$= \frac{981 m.L}{a.e} \text{ (for small deformations).}$$

[1] See p. 255.

Table II (*cont.*)

Shear modulus: $E/3$ (for non-compressible materials) $= n = \dfrac{\text{shear stress}}{\text{recoverable strain}}$

$$= \frac{1/3 \times \text{mass} \times 981}{\dfrac{\text{elastic stretch}}{\text{original length}}} = \frac{981m.L}{3a.e} \text{ (for small deformations).}$$

Viscosity: $$\eta = \frac{\text{shear stress} \times \text{time of flow}}{\text{flow}} = \frac{1/3m.981.t}{a.f/L} = \frac{981m.L.t}{3a.f}.$$

(This is, of course, a mean viscosity during flow—η is actually rising all the time if S is constant.)

Time of relaxation: $$t_r = \eta/n.$$

Structural viscosity may be expressed as $V_s = \dfrac{\eta_{\text{low stress}}}{\eta_{\text{high stress}}}$.

This is not generally accepted as a dimensional definition—many workers prefer to write

$$\eta = \frac{981m.t}{3a.(f/L)^{V_s}}.$$

Internal viscosity (elastic after-effect):

$$\eta_i \text{ (mean value)} = \frac{\text{shear stress} \times \text{time occupied by elastic recovery}}{\text{elastic strain}} = \frac{S.t'}{e/L} = \frac{981m.L.t'}{3a.e}.$$

Work hardening:

$WH = (\partial\eta/\partial f)_S$ (at constant stress). Work hardening is here defined in terms of viscosity.

True hysteresis: $$H_T = \text{function of } \left(\frac{n \text{ falling stress}}{n \text{ rising stress}}\right).$$

Relaxation flow: $$f_r = \frac{f_{\text{intermittent stress}} - f_{\text{continuous stress}}}{f_{\text{continuous stress}}}.$$

Of these the two moduli (E and n), viscosity (η), time of relaxation (t_r) and internal viscosity (η_i) are dimensional definitions and well recognized as such. The remaining "definitions" are given in a form suitable for use in the present work. Their actual form is a matter of convenience for the particular field in which they are to be applied. The definitions of work hardening, true hysteresis and relaxation flow are thus arbitrary.

1. *Shear modulus of elasticity*

Consider a block of cheese (Fig. 1) initially of the dimensions indicated by A–A', and of length L and cross-section area a. Placing a weight of mass m on the cheese will compress it to the form B–B', whose height is $L-(e+f)$. After time t the weight is removed and the cheese finally assumes the form C–C', thus recovering a height of e, f being the flow or non-recoverable deformation and e the recoverable deformation or elastic recovery (Figs. 2 and 3).

Then, shear modulus $= \dfrac{\text{Young's modulus}}{3}$ (Poisson's ratio being 0·5)

$$= \frac{\text{loading stress}}{3 \times \text{strain}} = \frac{1}{3}\frac{mg/a}{e/L} \text{ (for small strains)}$$

$$= \frac{mgL}{3ae}.$$

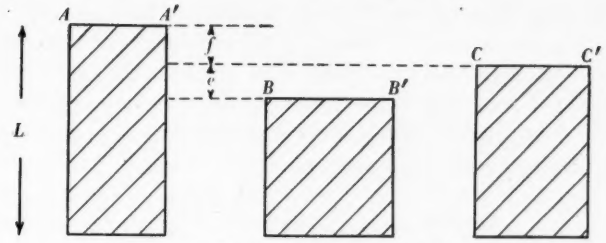

Fig. 1. Compression and elastic recovery of cheese plug.

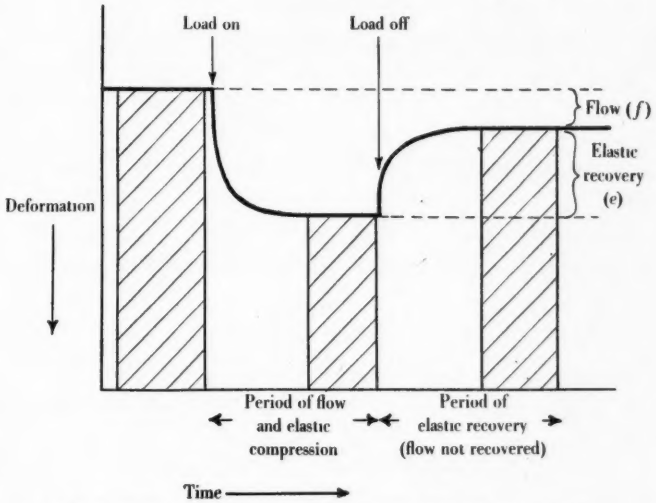

Fig. 2. Showing construction of deformation (strain)—time curve.

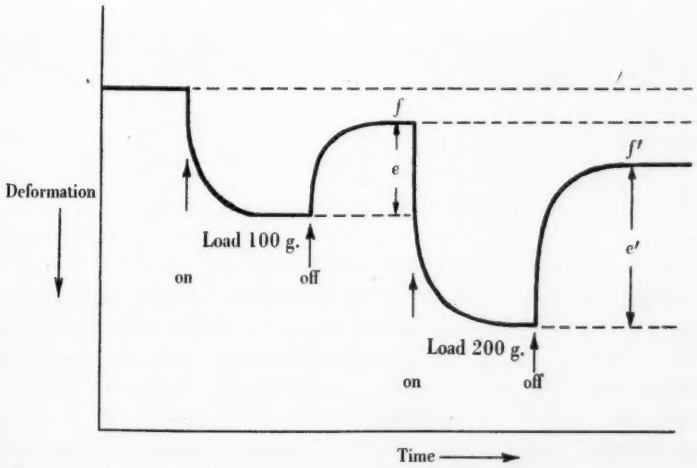

Fig 3. Showing recoverable and non-recoverable deformations produced by increasing loads.

The smaller this value (hereafter termed *modulus*) the more elastic is the cheese for a given viscosity. Under high stress-time conditions the elastic recovery of Cheddar cheese takes place in two phases, the first rapid (lasting about 15 sec.) in which the rate of recovery is inversely proportional to the amount recovered, and the second a very slow recovery (several minutes), the rate of which is approximately uniform.

2. *Viscosity*

The non-recoverable deformation f is a function of the viscosity of the cheese (Figs. 2 and 3).

$$\text{Mean viscosity during compression} = \eta = \frac{\text{shearing stress}}{\text{rate of deformation}}$$

$$= \frac{1}{3}\frac{mg/a}{f/L.1/t} = \frac{mg.L.t}{3a.f}.$$

The higher the viscosity the firmer or harder is the cheese for a given modulus.

3. *Time of relaxation*

This property (so-called by Maxwell) is given by the formula

$$t_r = \frac{\text{viscosity}}{\text{shear modulus}}$$

and so represents the ratio of viscosity to modulus.

It will be shown in the next paragraph that this is related to the *springiness* of the cheese.

4. *Internal viscosity* (*elastic after-effect*)

The behaviour of a plastic such as cheese may be simulated by the simple model shown in Fig. 4.

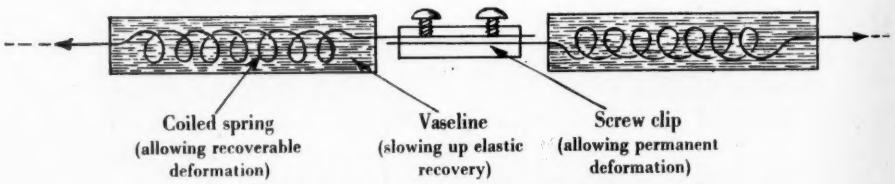

Fig. 4. Model illustrating behaviour of cheese.

For the purpose of illustration cheese may be considered to consist of numerous units of springs embedded in vaseline and joined together with screw clips. Extension of the springs to a given length for a given time will result in a state of extension consisting of (1) spring extension (elasticity) and (2) slight "give" at the screw clip (viscous flow). On removal of the force causing the extension, the springs will recover to their original length but rather slowly, due to the viscosity of the vaseline. This retarding effect of the vaseline is an illustration of elastic after-effect produced by *internal viscosity*.

The "give" at the screw clip is not recovered and represents permanent deformation which is a function of the viscosity.

It is clear that, if the springs are tough (high modulus), in order to produce a given deformation, a big stress and consequently much slippage will occur at the screw clip. The same effect is produced if the friction at the clip (representing "external" viscosity) is low, hence a very springy cheese will have a relatively low modulus or high elasticity.

Internal viscosity may here be defined by the formula

$$\eta_i = \frac{\text{shearing time} \times \text{time occupied by elastic recovery}}{\text{elastic strain}}.$$

The two phases of recovery in Cheddar cheese indicate two kinds of elastic after-effect, the later recovery perhaps being due to distortion of a more deep-seated nature than the earlier.

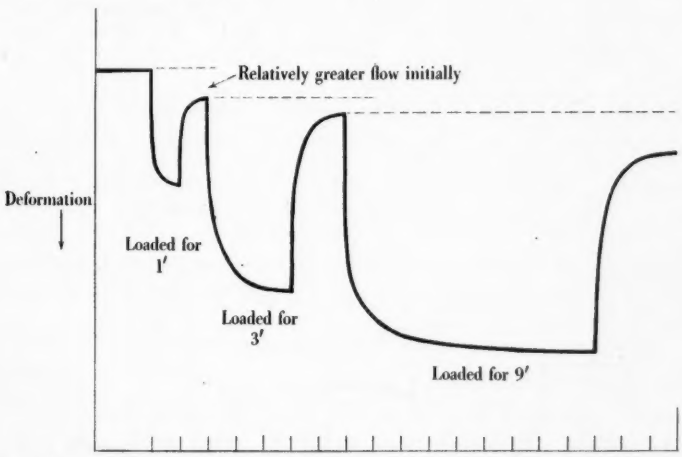

Fig. 5. Illustrating work hardening in cheese (i.e. change in rate of flow with flow).

5. *Structural viscosity*

This is a measure of the change of viscosity with stress. The change of η with rising stress is believed to give a measure of *brittleness* (Fig. 3). A high structural viscosity (i.e. fall of viscosity with increasing stress) usually indicates brittleness (5).

6. *Work hardening*

This is measured by the variation of viscosity with extent of deformation *at constant stress*, i.e. change in the rate of flow with flow (Fig. 5). Cheese and butter appear to exhibit appreciable work hardening.

7. *True hysteresis*

A convenient illustration of this property is a rusty spring which slides on a rod and does not return to the same position after extensions on both sides

of any arbitrarily selected position (Fig. 6). Thus a load W, giving the initial extension, is followed by a load of $2W$ and once more by a load of W. If hysteresis is absent the spring will be in its original position; the change in position is a measure of the true hysteresis(7) (cf. also(10)). The deformation-time curve obtained in this type of experiment is illustrated in Fig. 7.

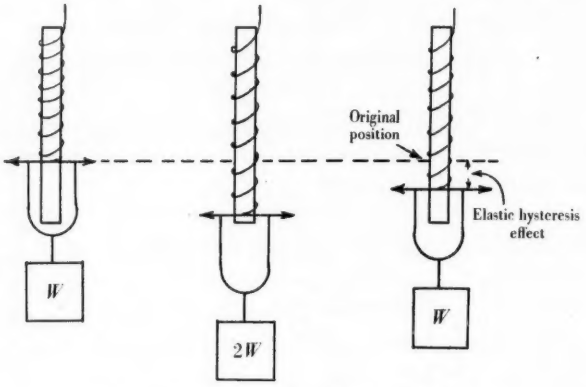

Fig. 6. Model illustrating elastic hysteresis effect.

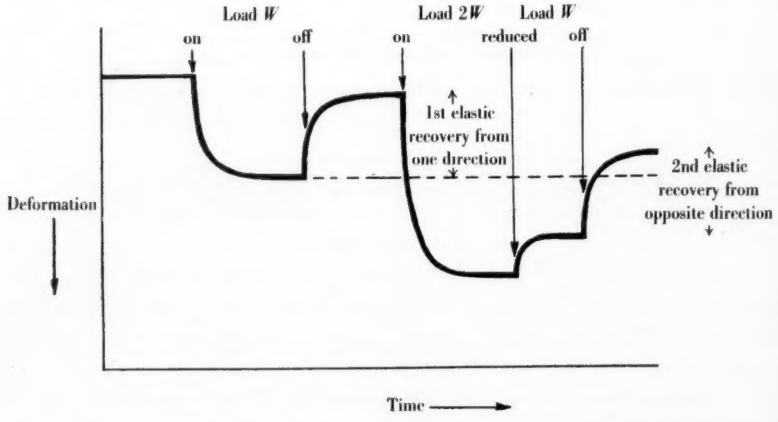

Fig. 7. Hysteresis curve obtained by measuring elastic recoveries from opposite directions.

In the analogy of the springs working in vaseline a hysteresis effect may be obtained by embedding sand or grit in the vaseline. By getting between the coils of the springs, the grit prevents the attainment of a true equilibrium just as the rusty spring sliding on a rod fails to return to the same position.

8. *Relaxation flow*

The flow which may occur during elastic recovery may be determined by straining in two stages, e.g. under a constant load, first without allowing an intermediate elastic recovery and then allowing recovery intermediately, the total strain being the same in both cases(7) (Fig. 8).

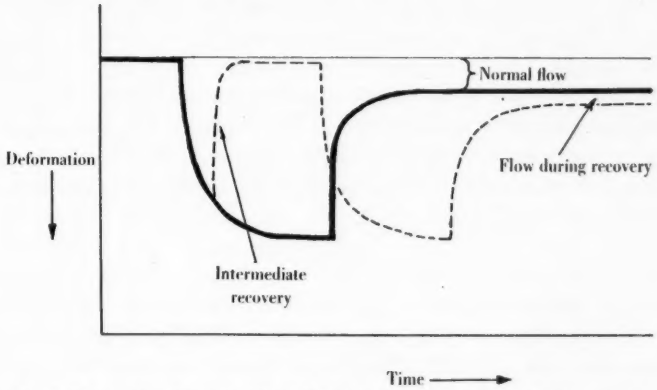

Fig. 8. Illustrating determination of flow during relaxation or elastic recovery.

EXPERIMENTAL TECHNIQUE

Sufficient accuracy in measurement may be obtained by a quite simple apparatus constructed with Meccano parts or similar material.

DESCRIPTION OF METHOD AND APPARATUS

The apparatus consists of a horizontal thin metal disk fixed to a rod which slides in a vertical bearing. To an adjustable collar on the rod is attached by a swivelling pin a long piece (44 cm.) of untempered wire or other suitable material. A cylinder or block of cheese 3–6 cm. long and 1·5–3 cm. in diameter

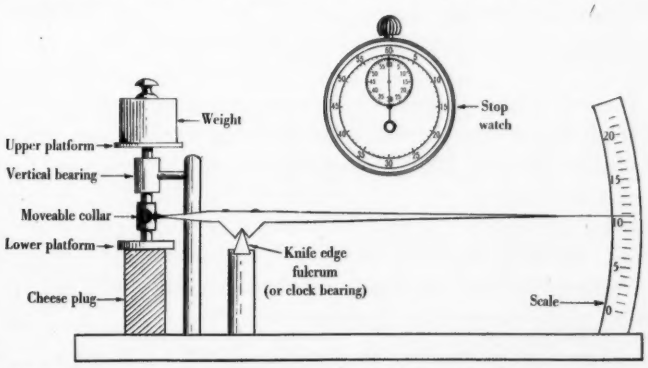

Fig. 9. Rheometer or apparatus for measuring deformations in cheese, butter and similar materials.

is placed at one end of the apparatus. The larger the plug the more accurate the values obtained as the sampling error is reduced and larger deformations are obtained. Moreover, frictional losses in the apparatus become less significant. If, however, ordinary borers must be used a plug about 3 cm. long and 1·7 cm. diameter is suitable. The platform (or disk) rests on the cheese plug and the wire passes over a knife-edge fulcrum about 4 cm. from the adjustable collar to give a scale reading at the other end of the wire magnifying the real deformation by about 10. A clock bearing may be used instead of a simple fulcrum if desired. Readings are taken to the nearest $\frac{1}{4}$ mm. A simple factor gives the real deformation from the scale reading. Cheese experiments should be made at a temperature of 18° C. ± 1° (65° F. ± 2°). The apparatus should be checked for blank values by using a cylinder of steel or other suitable material. With the loads used for butter, cheese, and similar materials the blank values are usually negligible.

Method

The apparatus is adjusted as described and a weight of 100–200 g. placed on the cheese for 5 sec. to ensure that a firm contact is established. The weight is then removed and readings taken until a constant value is obtained. A weight W (e.g. 200–500 g.) is then placed on the platform for a definite time (e.g. 60 sec.) and readings taken after 10, 20, 30 and 60 sec. The weight is then removed and readings similarly taken during recovery. Deformation values plotted against time give curves similar to those shown in Figs. 3, 5, 7 and 8.

A standardized plug of cheese may conveniently be cut off from a bore by a guillotine prepared by fixing two safety razor blades into a holder, e.g. at a distance of 3 cm. apart.

All the properties discussed in this paper (pp. 248–53) may be investigated quantitatively by this simple apparatus.

Among its advantages may be mentioned:

(1) It permits a purely objective measurement of those elastic and plastic properties which constitute "body".

(2) These can be recorded in absolute units and thus a permanent record made.

(3) The values can be compared by correspondence.

(4) It gives reproducible values, limited only by the "sampling error". This is present, however, in all cheese tests and may be minimized by testing three pieces of cheese.

(5) Other plastic milk products such as butter can be graded for "body" using similar methods and apparatus, e.g. the "spreadability" or viscosity of butter can be measured in this way.[1] Temperature control is, of course, of much greater importance when working with butter.

[1] The modulus values for butter are, relative to the viscosity values, so high that "spreadability" may safely be assumed inversely proportional to viscosity, although it may depend on V_g as well.

(6) Changes taking place in the curd during ripening may be followed by rheological methods and correlated with cheese type and chemical analyses. The effect of variations in methods of manufacture and of experimental conditions may also be investigated.

Practical aspects—the problems of grading

It is evident that the elastic and plastic properties described are closely linked with what the grader terms *body* and possibly also *texture*. Before proceeding to discuss their relationship it is necessary to emphasize that "elasticity" as a term commonly used really describes the *springiness* (*time of relaxation*) of a material and so is rather different from *modulus*. Thus a material of low *modulus* but high *internal viscosity* (*elastic after-effect*) would not be considered as elastic as a material of equally low *modulus* but with a low internal viscosity (Fig. 4). *Modulus* may thus be considered as a measure of *hardness in response to rapidly applied forces*.

Viscosity may be regarded as *ability to resist persistent loads, including gravity*.

The interrelationship of practical tests and the conditions controlling plastic behaviour are summarized in Table III. *True hysteresis* and *relaxation flow* are not included in this table. Their importance lies chiefly in revealing the likely errors (and so the probable accuracy) of the experimental methods employed.

Table III

Test	Sensation or result	Rheological properties concerned
1. Pressing with thumb into cheese, momentarily holding and then removing	(i) Hardness	The sum of a function of *viscosity* and *modulus* (chiefly the latter for hard cheese)
	(ii) Spring (i.e. the amount of recovery)	*Time of relaxation*
	(iii) Speed of recovery	*Elastic after-effect*
2. Applying a constant *force* against the cheese	Increase in hardness as test proceeds	*Work hardening*
3. Pressing with different forces	Amount by which cheese feels softer when it is pressed harder	*Structural viscosity*
4. Ordinary crushing	Crumbliness ("shortness")	*Structural viscosity* (?)
5. Pressing on cheese (e.g. by a weight) for some time, removing and allowing full recovery	Permanent change in shape	*Viscosity* (external)
6. Applying increasingly heavy loads	(i) Load required to crush	Crushing or compressive strength
	(ii) Extent of deformation before crushing	Plasticity (?)

The measurement of *body* in cheese

For practical purposes it is probably sufficient to make two simple determinations:

(1) *Viscosity* and *modulus*. For this purpose the second phase of slow recovery may be ignored and an arbitrary period of recovery allowed, e.g. 30 sec. for deformation and 30 sec. for recovery. Only the final values need be

recorded, i.e. after 30 and after 60 sec. The values obtained will naturally differ from the true values by a fairly constant amount. *Modulus* values will be slightly high and *viscosity* values low. The error is negligible with low stress-time conditions. This is illustrated in Fig. 10.

In Fig. 10 e and f represent the apparent elastic recovery and flow after 30 sec. when a large stress-time condition has been applied. The true values e' and f' are only obtained by waiting several minutes for the system to come to equilibrium.

Instead of reading the deformation visually it may be more convenient, especially when carrying out a large number of tests, to attach a light quill pen to the end of the lever and let the deformation-time curve record itself, as on a barograph. In this way direct records may be obtained. It is necessary

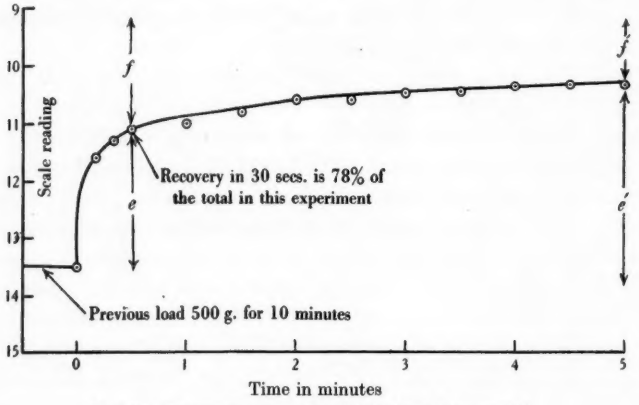

Fig. 10. Recovery curve after high stress-time period.

in this case to use a light lever in place of the untempered wire and to see that friction of the pen on the rotating graph paper is reduced to a minimum. Large plugs, e.g. 6 by 3 cm. diameter, will naturally reduce the sampling error and make for easier reading of the curves but entail the taking of specially large bores. It may be advantageous to use a doubled-jointed beam to magnify the deformation to a greater extent (see Fig. 11).

(2) *Crushing strength.* This may be conveniently determined by allowing a very heavy weight, e.g. 1000 g., to hang from a spring balance and rest on a sliding platform and cheese plug. By lowering the spring gradually and at a constant rate with a winch, increasing loads are put on to the cheese. The load required to crush the plug is recorded. "Crushing" is rapid flow.

The load on the cheese is, of course, the amount of the weight used (1000 g.) minus the reading on the spring balance (Fig. 12). The result will depend on the rate at which the load is increased; it is important therefore to standardize this.

It must be remembered that viscosity and other values are only mean

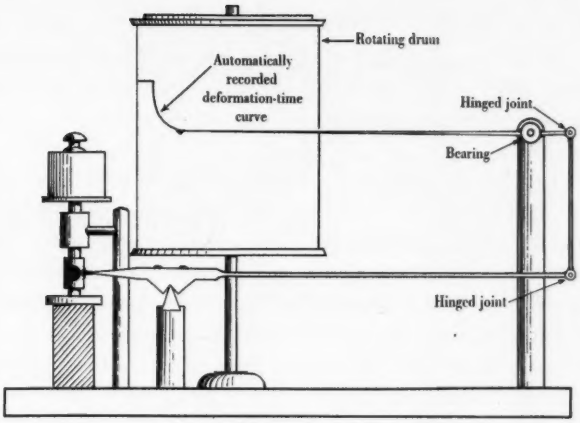

Fig. 11. Self-recording rheometer.

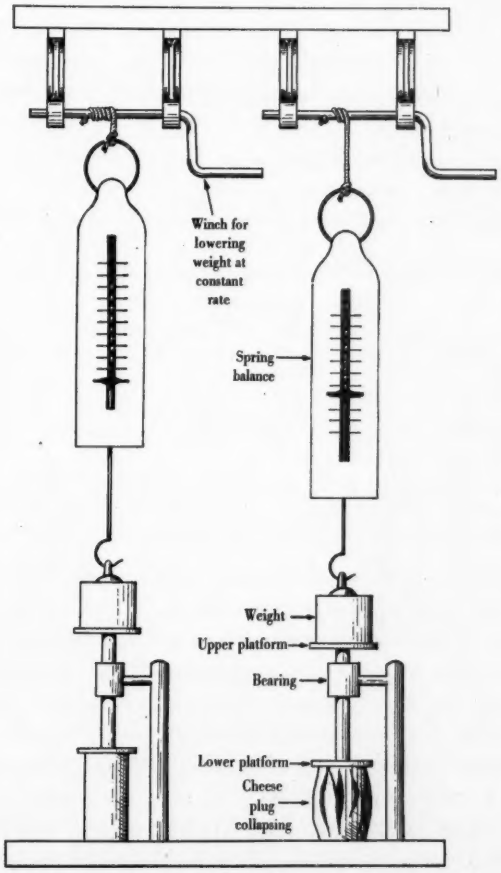

Fig. 12. Apparatus for determining the crushing strength of cheese.

values holding for the conditions of the experiment. When comparing cheese, etc., therefore, plugs of approximately the same size should be used and time-stress conditions should be identical.

The measurement of *texture* (openness)

It can be shown by experiment that viscosity values obtained by the first of any series of loadings, e.g. with 100 g. for 1, 3 and 9 min., 200 g. for 1, 3 and 9 min., etc. are always appreciably lower than those obtained with subsequent loadings (cf. Fig. 5). This is a *work hardening* phenomenon. It would appear possible, however, in view of what is known of the nature of cheese, that this relatively greater flow is due to the air or gas spaces in the cheese mass and that the apparently greater flow is due to the filling up of these spaces, which could obviously only be filled once. If this explanation is true the phenomenon could be utilized as a measure of openness or *texture*. This is, however, at the present, only a hypothesis.

Experimental results

Determinations of apparent *viscosity* and *modulus* have been made on different varieties of cheese.

I. Through the courtesy of the British Dairy Farmers' Association it has been possible to make measurements on the 1st prize cheese at the London Dairy Show, 1936. The results are given in Table IV, and the analyses of the

Table IV. *Modulus and viscosity values of B.D.F.A. prize cheese, Dairy Show*, 1936

Cheese	Viscosity	*pV*	Shear modulus	*pM*	*pS*
Cheddar	141×10^6 g./cm./sec.	8·15	$1{\cdot}15 \times 10^6$ g./cm./sec.^{2}	6·06	2·09
Dunlop	167 ,,	8·22	1·13 ,,	6·05	2·17
Gloucester	155 ,,	8·19	1·15 ,,	6·06	2·13
Derby	110 ,,	8·04	1·07 ,,	6·03	2·01
Cheshire	56 ,,	7·75	0·79 ,,	5·90	1·85
Leicester	35 ,,	7·54	0·50 ,,	5·70	1·84

These results are the mean values of experiments on 2 or 3 plugs about 3 cm. long by 1·7 cm. diameter and using loads of 100 and 200 g., i.e. 4 or 6 results. The results obtained initially with a load of 50 g. were in most cases significantly lower than the mean values obtained with 100 and 200 g.

cheese in Table V. In these experiments a fixed time of 30 sec. both for compression and recovery was allowed. Hence the values are described as "apparent" although under these conditions the error is insignificant. If high stresses are allowed to act for long times (cf. Fig. 10) the error becomes significant.

The six types of cheese examined are put into the following order of decreasing *body* by practical experts: (1) Cheddar, Dunlop and Gloucester, (2) Derby, (3) Cheshire, (4) Leicester.

It will be seen in Table IV that both the *modulus* and *viscosity* values place the cheese in the same sequence. There is thus good agreement between rheological properties and practical grading.

Table V. *Analyses of B.D.F.A. prize cheese*

Age ...	Cheddar 3 months	Dunlop —	Gloucester 2 months	Derby 2 months	Cheshire 1 month	Leicester 2 months
			% of cheese			
Moisture	34·08	34·45	35·77	35·61	38·24	39·62
Fat	35·1	33·6	36·6	34·2	31·7	30·0
Salt	1·60	1·73	1·86	1·85	1·83	1·50
Acidity (as lactic acid)	1·94	1·81	2·00	1·99	2·06	2·07
Ca	—	0·756	0·787	0·880	—	0·824
Total nitrogen	3·85	3·910	3·506	3·553	3·589	3·712
			% of total nitrogen			
Non-protein N	16·2	19·9	19·1	26·7	17·0	16·6
Total Sörensen N	5·0	8·6	10·8	16·7	4·7	10·0
Non-protein Sörensen N	4·2	6·2	7·9	12·8	3·5	8·3
Ammonia N	0·96	1·5	2·6	4·0	1·2	2·8

The rheological properties clearly show that Cheddar, Dunlop and Gloucester are relatively firm cheese. Derby is intermediate and Cheshire and Leicester are much softer. Fig. 13 shows the actual curves obtained with Gloucester cheese using three different plugs. The agreement between plugs is, in this case, rather better than the average.

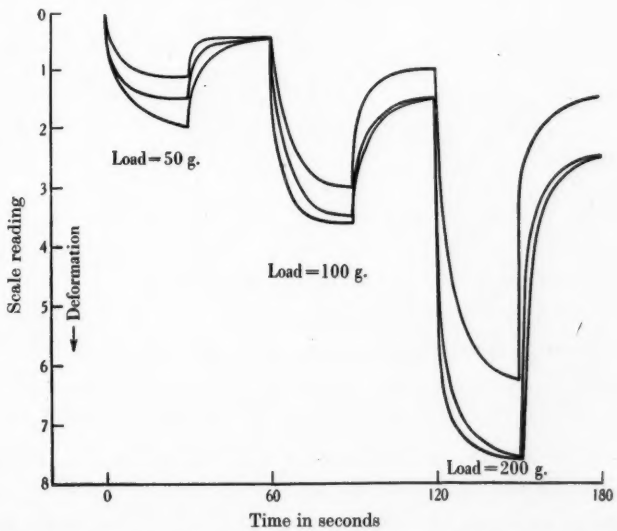

Fig. 13. Deformation curves obtained with three plugs of B.D.F.A. prize Gloucester cheese.

II. Similar determinations have been made on different types of cheese all made on one day from the same milk at this Institute. These cheese were kindly placed at my disposal by Dr A. T. R. Mattick and Mr J. Harrison. The deformation-time curves are shown in Fig. 14. A similar sequence of hardness will be evident. Lancashire is appreciably less viscous than the other three.

III. *The effect of mastitis on cheese body.* This is dealt with elsewhere(11) where it is shown that cheese made from mastitis milk may be significantly weaker in body (less viscous) than that from non-mastitis milk. There is a greater flow in the case of the mastitis cheese, but the elastic recoveries are not greatly different.

IV. *The effect of time and stress on the recovery curve.* The composite nature of this curve has already been discussed. Allowing only 30 sec. for recovery in experiments involving low time and stress factors will not significantly affect the result. If a high load is allowed to act for some time (e.g. 10 min.) only about 80 per cent of the recovery may be effected in 30 sec. (Fig. 10). Thus in experimental work (as distinct from practical grading) recovery should be allowed to proceed until three identical readings are obtained.

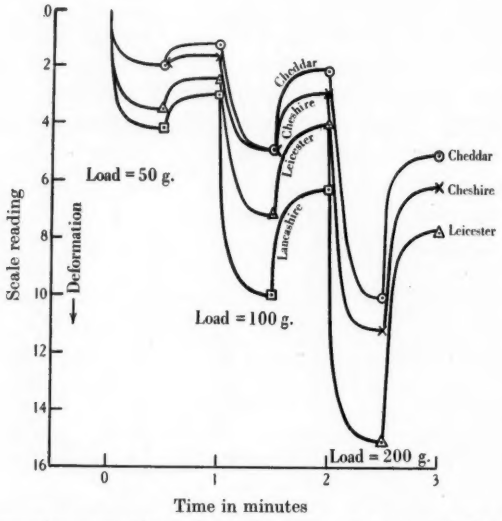

Fig. 14. Deformation curves obtained with four types of cheese made from the same milk.

Units

The unit of viscosity is the *poise.* A value of 10^6 poises may therefore conveniently be referred to as 1 *megapoise.* In view of the wide variations found in dairy products, e.g. between cheese and butter, it is more convenient to express body as the logarithms of the modulus and viscosity in absolute (c.g.s.) units. Thus pM = logarithm of the shear modulus in g. cm.$^{-1}$ sec.$^{-2}$, and pV = logarithm of the viscosity in g. cm.$^{-1}$ sec.$^{-2}$. This permits the use of a very compact figure. It should be remembered, however, that the scale is logarithmic and that a difference of 0·3 in pM or pV units indicates a doubling or halving of modulus or viscosity. A difference of 0·05 (representing about 12 per cent) may be significant in cheese and is certainly significant for butter.

This system also allows the calculation of Maxwell's *time of relaxation* value logarithmically ($\log t_r = pV - pM$). This value may be expressed similarly as pS, the S here standing for springiness, so that $pS = pV - pM = \log t_r = \log \frac{\eta}{n}$. Thus the higher the pV the harder the material, the higher the pM the *less* elastic, and finally the higher the pS the more springy is the material.

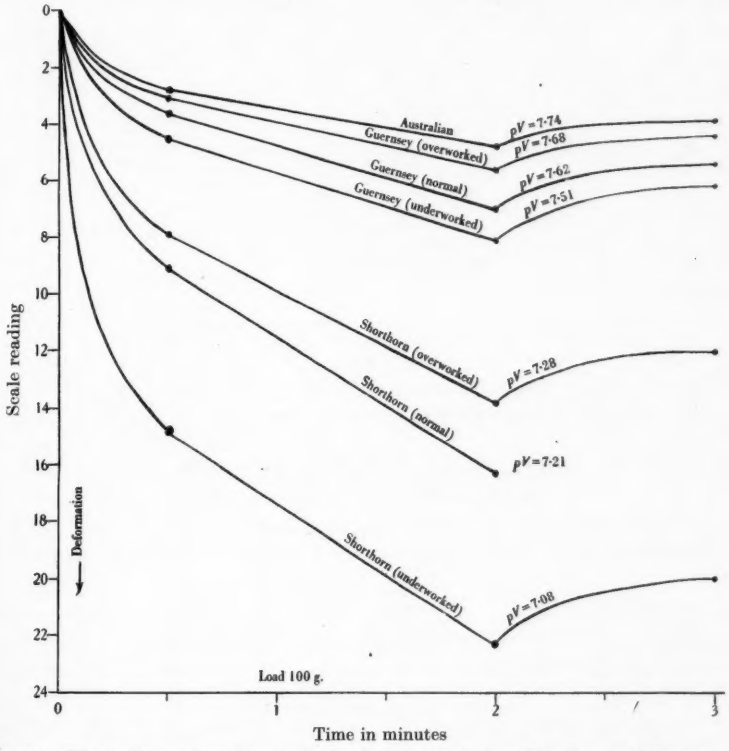

Fig. 15. Effect of time of working on body of butter made from Shorthorn and Guernsey milk. (Plugs 2·6 cm. long × 2·27 cm.² cross section; temperature 16–17° C.)

BUTTER

The apparatus as described for cheese is quite suitable for use with butter. Temperature control is, however, of far greater importance than with cheese. While a temperature of $18 \pm 1°$ C. is quite suitable for cheese, a much closer control is necessary for butter. A temperature of $15{\cdot}5 \pm 0{\cdot}1°$ C. is suggested. Where this is impossible it is essential at least to compare butters at the same temperature.

Typical results are given in Table VI and in Figs. 15 and 16.

In Fig. 15 are shown the deformation curves obtained by allowing a weight of 100 g. to compress plugs of butter at 16–17° C. It will be seen that butter

made from Guernsey milk is very much firmer than that made from Shorthorn milk under the conditions of the experiment and that the extent of working exerts a marked influence on the viscosity.

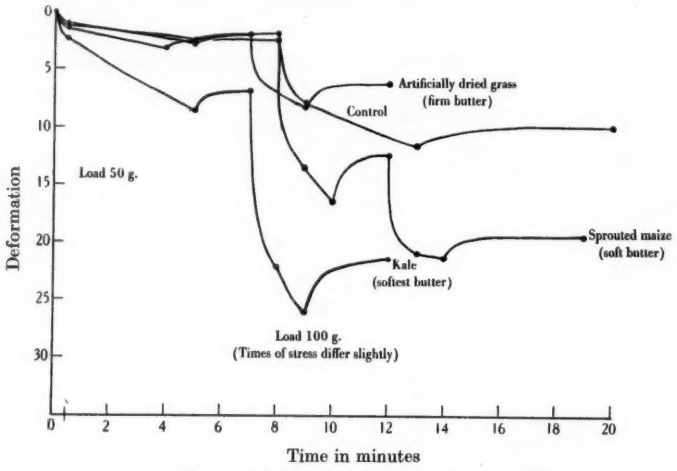

Fig. 16. Effect of different feeds on the viscosity of butter.
(Plugs 3 cm. long × 6·5 cm.² cross section; temperature 16–17° C.)

Table VI. *The effect of working on viscosity and modulus of butter made from Shorthorn and Guernsey milk*

Milk	Working	*pV*	*pM*	*pS*
Shorthorn	Underworked	7·08	5·98	1·10
,,	Normal	7·21	—	—
,,	Overworked	7·28	6·17	1·11
Guernsey	Underworked	7·51	6·02	1·49
,,	Normal	7·62	6·12	1·50
,,	Overworked	7·68	6·22	1·46
— (Australian)	—	7·74	6·39	1·35

The effect of foodstuffs on butter

Feed	*pV*	*pM*	*pS*
Normal winter (control)	7·16	5·66	1·50
,, +artificially dried grass	7·24	5·66	1·58
,, +sprouted maize	6·89	5·34	1·55
,, +kale	6·75	5·28	1·47

These two sets of values are not comparable one with the other.

Working is known to decrease progressively the size of the water globule and to this phenomenon may be attributed not only the increase in viscosity but also the improved keeping quality consequent on the lessened rate of bacterial proliferation (12). The *pS* values for Shorthorn and Guernsey milk appear to be significantly different.

In Fig. 16 are given deformation curves for butter made from milk of cows maintained on different feeds. Artificially dried grass results in a much firmer butter than either sprouted maize or kale.

VISCOSITY, MODULUS AND SPRINGINESS

The relation between these three properties is shown graphically in Fig. 17 where flow and corresponding viscosity values have been plotted against elastic recovery and corresponding modulus values. Logarithmic scales have

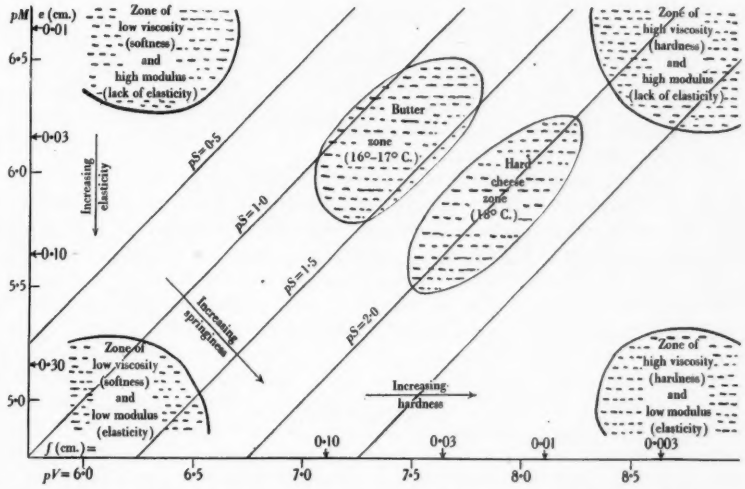

Fig. 17. Graph showing the relationship between modulus or elasticity (e and pM), viscosity (f and pV), and springiness (pS). Plugs 3 cm. long by 1·7 cm. diameter, load 100 g., time of stress 30 sec.

been used so that pS values lie on oblique straight lines. By drawing these lines for given pS values and reading off the experimentally determined pV and pM values the pS value is obtained, and the general characteristics of the material readily appreciated. Zones indicating the order of value obtained with hard cheese and butter have been drawn. The four corner zones represent materials having extreme properties and show, for example, how for a given modulus viscosity may affect springiness. This diagram epitomizes the plastic and elastic properties not only of cheese and butter but of all similar materials.

ACKNOWLEDGEMENTS

The writer very gratefully acknowledges the valuable criticism and many helpful suggestions of Dr G. W. Scott Blair, of the Rothamsted Experimental Station. He is also indebted to Dr W. L. Davies for the cheese analyses and to Miss D. V. Dearden for churning the butters.

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(*MS. received* 20 *May*, 1937)

Note added 28th June, 1937.

Further investigation has emphasized the importance of moisture (total and free) in controlling viscosity and modulus in cheese. In hard cheese there exists a gradient in viscosity and modulus which increases from the interior outwards. A similar gradient has been observed in the rate of ripening and in oxidation/reduction potential. It is apparently due to the greater rate of evaporation from the portion near the surface. The sampling errors are largely due to this fact. It is important therefore when determining pV and pM values to make allowance for this by taking plugs at a constant depth (or depths), *e.g.* beginning at 2 in. beneath the surface of the cheese.

Methods for estimating bound moisture in cheese are being investigated in collaboration with Dr G. W. Scott Blair.

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BIENNIAL REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

SECTION F. MILK-BORNE DISEASES

In my previous biennial review(1) on these diseases, attention was directed especially to the bacteriological technique required for the identification of the specific causal bacteria in the cow and especially in its milk. In the present review I propose to refer to the epidemiology of milk-borne infections, so far as the inadequate data permit. But some points of technique additional to those in the previous review first require discussion.

I. The streptococcal infections

The methods previously described, especially the precipitin tests for the "groups" A, B, C, etc. of Lancefield and the slide-agglutination tests for the "types", 1–27, of Griffith within group A (comprising the great majority of the haemolytic streptococci pathogenic for man), have established themselves on the basis of further experience and no change in their conduct has to be recorded. The correlation of types determined by agglutination with those defined by precipitin tests (*M* substance) and by protection tests on animals is being actively investigated, especially in America. On the whole, they are satisfactorily concordant and indicate that Griffith's types are based on properties of pathogenic and, especially, of epidemiological significance as, indeed, the facts adduced by Griffith as to their distribution in human disease most strongly suggested. The only other contribution of possible taxonomic importance and practical value is that of Evans(2) on the existence of groups of streptococci determinable by phage-sensitiveness. How far such phage types coincide with those already established by serological tests is not yet clear and must remain in doubt until the technique of phage typing among the streptococci becomes capable of greater precision. Depending, as it does in most cases, on temporary lysis during the early stage of broth culture alone, the identification of streptococcal phages has not yet attained the exactitude observable with, for example, the pathogenic bacteria of intestinal habitat. Their importance in the pathology of infection and in preventive medicine is, of course, at least as dubious as that of other phages.

II. The *Brucella* infections

Much work has been done on (1) the relative efficiency of direct culture and guinea-pig inoculation in the detection of brucellas in milk; (2) the cultural and serological differentiation of *Br. melitensis*, *Br. abortus* and *Br. suis*; (3) the minimum titres of agglutinin in bovine serum or milk whey which indicate *Brucella* infection among bovines; and (4) the laboratory methods to be used in creating or maintaining *abortus*-free herds and, especially, on the limitations which must be reckoned with in the efficiency of these methods.

As regards (1) Thompson(3) found guinea-pig inoculation only slightly better than direct culture on Huddleson's liver-extract-gentian-violet agar, examining quarter samples of milk at frequent intervals from ten infected cows. Karsten & Bischoff(4) examined 466 samples of milk by both methods and got a positive diagnosis in 101 by direct culture but in 184 by guinea-pig inoculation. Van Oyen(5), however, using serum-agar with 1 in 100,000 gentian-violet, states that culture was better than guinea-pig inoculation for detecting *abortus* in milk. It is evident that, though

culture is quicker and cheaper and, perhaps, equally efficient with suitable samples, guinea-pig inoculation cannot be omitted in searching for brucellas in milk.

As regards (2), Cumming(6) submitted twenty-three strains of each species, *melitensis*, *abortus* and *suis*, to five different laboratories for report as to cultural identification. Growth on thionin-agar and on basic-fuchsin-agar and production of H_2S by liver-agar cultures were used by all as differential features; cultures sensitive to thionin (in failing to grow in its presence) but not to fuchsin were labelled as *abortus*; those showing opposite sensitiveness as *suis* and those insensitive to both as *melitensis*; those producing H_2S in 2 days were noted as *abortus*, those requiring 4 days as *suis* and those producing at most a trace in 4 days as *melitensis*. There was close general agreement in the results of test for dye-sensitiveness but the production of H_2S was more irregular.

Veazie & Mayer(7) studied the serological behaviour of 400 *Brucella* strains. 10 per cent of those identified by cultural behaviour as *Br. abortus* were serologically *melitensis* but nearly half of these aberrant strains came from one (American) herd. 12 per cent of those identified culturally as *melitensis* were serologically *abortus* but these were all old laboratory strains of unknown origin; it has long been recognized that the biochemical characters of *Brucella* strains alter during prolonged subculture.

On the whole, the conclusions reached by Wilson(8) and by Wilson & Miles(9) still stand, namely, that no single test suffices to allocate individual strains of brucellas to their proper species, but that study of all the characters, cultural and serological, will, in general, leave no doubt as to the specific name of any strain, though subgroups can be distinguished on the basis of minor variations.

As regards (3) much discussion has occurred and no agreement is likely to be reached until the conditions under which agglutination tests are done have been standardized. A full statement of these conditions, along with a comparison of "official" tests in different countries, has been published by Stableforth(10, 11).

He points out that the tests vary to such an extent that one may be as much as five times more sensitive than another, due chiefly to differences in the opacity, i.e. in the bacterial content, of the agglutinating mixtures but partly to varying agglutinability of the culture employed. His recommendations for a standard technique of the agglutination test include (1) the preparation of the bacterial emulsion with an opacity corresponding to tube 2 of Brown's series, i.e. to that of a mixture of 3 ml. of 1 per cent barium chloride with 97 ml. of 1 per cent sulphuric acid; (2) the control of the inherent sensitiveness of the bacteria to agglutination by means of a standard (dried) agglutinating serum with which the emulsion must give the known titre under standard conditions; the standard serum referred to was obtained from a naturally infected cow and had a titre of + (=25 per cent clearing and sedimentation) with the standard emulsion in 20 hours at 37° C. (air incubation) at 1 in 480 dilution of the reconstituted dried serum; (3) the use of air incubators at 37° C. during the agglutination, since the temperature of 55° C. produces or intensifies "pro-zones", as does pre-heating of the serum, "inactivation", at this temperature.

As regards diagnostic criteria Stableforth(11) gives the following table for blood serum tests of cows suspected of *abortus* infection.

Agglutination	Cows in which condition as to pregnancy not known	Known pregnant cows
+ 1 in 40	Positive	Positive
+ + + 1 in 20	Positive	Positive if less than 3 months, otherwise doubtful
+ + 1 in 20	Positive	As above
+ 1 in 20	Doubtful	Doubtful if less than 1 month, otherwise negative
+ + + 1 in 10	Doubtful	As above
+ + 1 in 10	Doubtful	As above
+ 1 in 10	Negative	Negative

The sign + + + means complete clearing and sedimentation, + + means 50 per cent clearing and sedimentation, and + 25 per cent. These criteria apply only to sera tested in the manner described above. Examined, for example, by the technique of the U.S. Bureau of Animal Industry, in which a thin sensitive suspension is employed, the diagnostic titres would be much higher, 1 in 100 being roughly equivalent to the 1 in 20 of the table given above.

The numerous observations which have been made in the attempt to correlate agglutinin titres (blood serum or whey) in infected cows with the presence in their blood or milk of the specific bacteria are difficult to assess because of these technical variations. Van Oyen (5) gives the following figures in this connexion:

Cows	Whey agglutination	Milk culture positive
797	Negative	1
56	Titre 1–100	14
50	,, 1–200	24
33	,, 1–500	22

Karsten & Bischoff (4) examined 900 infected cows and conclude that whey agglutination gives 10–30 per cent fewer positives than blood serum agglutination (560 = 62 per cent as compared with 841 = 93 per cent). They compared also the results of complement-fixation tests on the same specimen and found a few positives in specimens negative to agglutination, the result of summing the two tests being to raise the total percentage of positives to 83 per cent in the case of whey examination and to 96 per cent in the case of blood serum. As regards (4), the limitations of blood serum or whey agglutination tests in the control of *abortus*-free herds, one of them which is becoming widely recognized is that negative reactors may become positive without any apparent possibility of an intervening infection. Cases of delayed appearance of a positive reaction have long been known among pregnant infected cows or heifers; 5–10 per cent of such animals may behave in this way, the positive result usually appearing after the parturition, whether premature or at full term. Karsten (12) cites the case of a herd of 240 cows, thirty-three of which had negative agglutination reactions both in blood and whey up to the time of parturition but gave cultures of *Br. abortus* from the foetal membranes. Of these twenty-one continued to give negative agglutination tests. Hayes & Barger (13) report similar findings, cows excreting *Br. abortus* for months with completely negative agglutination tests. Doyle & Beckett (14), however, though recognizing the existence and importance of this phenomenon, found only two cows excreting *Br. abortus* in their milk out of 309 which gave negative results to blood-serum agglutination tests at a titre of 1–25. They accept McFadyean's dictum that, in practice, a cow giving such a negative result is free from *abortus* infection. Stableforth (10), discussing the whole question of agglutinin testing in the attempt to create *abortus*-free herds, notes the case of five cows out of twelve found infected in an apparently clean herd in which consistently negative tests had been got until after parturition. The proportion of cases of this kind may evidently be much greater than was previously thought. No other routine test for the presence of *abortus* infection in dairy herds is at present practicable, though further exploration of the intradermal test is indicated. Stockmayer (15) has done valuable work on the persistence of udder infection, the effect of the sex cycle on excretion of *abortus* and the relative value of procedures for its detection.

III. Tuberculosis

The chief advance in the methods of examining milk for the presence of tubercle bacilli is in the recognition of the possibility of direct microscopical detection of tuberculous infection emphasized by Cowan & Maddocks (16). They point out that restricting the high-power examination of milk deposits to the endothelioid cell

groups facilitates the demonstration of acid-fast bacilli and confers on this demonstration absolute diagnostic significance. Mrs M. L. Cowan Maitland (17) has recently compared the results she has obtained by microscopical examination of quarter samples from 950 cows with the test by guinea-pig inoculation and with the results of skilled veterinary inspection. All her positives were confirmed by the guinea-pig and only in one instance—in which, though cell groups of tuberculous type were present, no acid-fast bacteria were found—did the guinea-pig detect tuberculous infection missed by the microscope. As regards the clinical examination, on nine farms udder tuberculosis was wrongly suspected by it in one or more cows, while on five farms the cows secreting tubercle bacilli (as determined by the microscope and confirmed by inoculation) were missed on clinical inspection. The advantages of early detection (within 48 hours of sampling) of tuberculous milk are so great, both to the farmer and to the public health, that the method is worthy of extended trial. Following up Matthews's (18) observation that slow spinning of milk produces better films for microscopical examination, the Manchester workers have determined that 3 min. at 2500 r.p.m. is the most suitable for 50 ml. quantities. For details of the making of films, the article by Cowan & Maddocks (16) should be consulted and for the precautions against adventitious occurrence of acid-fast bacilli the report of Wilson.

The relative efficiency of cultivation of tubercle bacilli direct from milk samples as compared with guinea-pig inoculation is still the subject of active investigation. Ruddock-West & Alpe (19), using a modified Löwenstein medium, maintain that culture is at least equal to inoculation in detecting tubercle bacilli in milk from individual cows. Plum (20), using a similar method, had rather more irregular results; in comparing cultures from tuberculous uterine discharges with guinea-pig inoculation of the same material, the latter gave seventy-one positives as compared with thirty-seven by culture. Plum's results with microscopical examination as contrasted with culture are also worth notice; of bovine lung secretion microscopically positive, only 85 per cent gave cultures of tubercle bacilli; on the other hand, 25 per cent of specimens microscopically negative were culturally positive. In the case of udder secretions 96 per cent of those microscopically positive were also culturally positive, while 9 per cent of those microscopically negative nevertheless gave tubercle bacilli in culture.

Mattick & White (21) draw attention to the necessity of inoculating at least two guinea-pigs with each milk sample. Among 1000 samples of bulk milk inoculated into a single guinea-pig, eighty-four would require reinvestigation owing to the premature death of the inoculated animal and sixty-two specimens would be falsely returned as negative, whereas when inoculated into two guinea-pigs only seven of the 1000 specimens would require repetition and only four would give false negatives.

Eickmann & Schopen (22) have found treatment of bovine faeces with potassium bichromate (5 g. faeces in 30 ml. of 1 in 250 solution for 20 hours) of value in detecting tubercle bacilli in such material, the deposit being inoculated into guinea-pigs.

IV. The *Salmonella* infections

No technical advances of importance fall to be recorded in the detection of salmonellas except that Kauffmann's (23) combined brilliant-green-tetrathionate medium has been everywhere found to be superior to all other "enrichment" media for the isolation of salmonellas, including the typhoid and paratyphoid bacilli, from faeces, foodstuffs, etc. It may be mentioned that Wilson-Blair bismuth-iron-sulphite agar, which is invaluable in the detection of typhoid bacilli in such difficult material as sewage or milk, is procurable in powdered form under the Difco brand: this product has proved thoroughly satisfactory in field work and is very convenient.

New serological types in the *Salmonella* group continue to be described but none of these appear to have special association with cow's milk. Some remarks on the laboratory investigation of salmonellas will be found in a circular Med. 188 issued by the Ministry of Health and obtainable from H.M. Stationery Office. It is suggested that the precise identification of type need not be attempted by routine public health laboratories except in the case of the commoner ones, such as *S. typhi murium* (*aertrycke*) and *S. enteritidis* (*Gaertner*) but that the less common types should be submitted to laboratories such as that of the Ministry of Health or the Statens Serum Institut, Copenhagen, which possess a full range of type sera.

V. *Staphylococcus* infections

Much attention has been paid in the last 5 years to *Staphylococcus* infection of food-stuffs as a cause of acute gastro-enteritis, and the presence of staphylococci in milk can no longer be regarded as insignificant, however commonly they occur. The conditions under which such infection of milk confers on it pathogenic properties are still imperfectly understood but that it can do so is now beyond doubt.

The growth of certain strains of staphylococci in milk and other culture media, including, especially, artificial creams (largely farinaceous) and cooked gelatinized meat, results in the production of a heat-stable toxin, not identical with the skin-necrotizing toxin or the haemolysin previously known to exist in *Staphylococcus* cultures. Its effect, as shown on administration by the mouth to monkeys or to human volunteers, is to set up acute irritation of the stomach with vomiting in from 30 min. to 4 hours, followed in many cases by diarrhoea. The symptoms, though violent, are not prolonged and the death-rate is very low. Crabtree & Litterer(24) were apparently the first to associate a milk-borne outbreak of acute gastro-enteritis with the presence of *Staph. aureus* in the milk. There was no sign of acute mastitis in the two cows concerned but cultures from their milk showed large numbers of *Staphylococcus* colonies. The milk produced seven outbreaks in succession in a community of ninety-seven persons, the total number of attacks being 242; the deciding factor as to whether a particular day's milk should be toxic appeared to be the temperature at which the milk was kept before consumption. Shaughnessy & Grubb(25) have described a similar outbreak affecting 25 persons in which the milk came from cows suffering from *Staphylococcus* mastitis and they suggest that this form of mastitis, common though it is, may not be so innocuous to human consumers of the milk as was formerly supposed. In the *Report of the Chief Medical Officer of the Ministry of Health* for 1935, p. 147, reference is made to a British outbreak in which staphylococci found in milk and in a custard made with it, responsible for cases of acute gastro-enteritis, produced in broth culture a highly potent toxin, 10 ml. sufficing to produce vomiting in a monkey to which it was administered by stomach tube. There can be no doubt that some, at least, of the outbreaks of acute vomiting with rapid recovery which have been observed in the past in association with the consumption of particular batches of milk were due to this form of milk infection. Technically the demonstration of the toxin in milk or other food-stuffs is difficult, since monkeys and human volunteers appear to be the only susceptible animals. Gross infection of suspected milk with staphylococci would, however, furnish a prima facie case corroborating the suspicion. Dolman *et al.*(26) have suggested that the specific gastro-enteritis toxin can be detected by its emetic effect on kittens injected intraperitoneally with small doses (2 ml.) of filtrate, heated for half an hour in boiling water to destroy the other toxins. "Cheese poisoning", which has long been bacteriologically an unsolved problem, may be an example of the effects of *Staphylococcus* toxin and research to settle this is desirable.

VI. Epidemiology

The epidemic milk-borne diseases have commonly been divided into two groups: I, those in which milk transmits to man a disease normally affecting the bovine species, e.g. bovine tuberculosis, brucellosis, and, more rarely, salmonellosis, and II, those in which the infection of a human disease accidentally enters milk between milking and ingestion by the human consumer, e.g. the intestinal infections—including those of enteric fever, paratyphoid fever, food poisoning, and bacillary dysentery in its several varieties—and the throat infections, including diphtheria (uncommon but indubitable), scarlet fever and non-eruptive tonsillitis. A review of the history of both these groups with numerous examples has recently been published by Savage(27). In addition to group II, as defined above, a further category, group III, of specifically human infections can be distinguished among those conveyed by cow's milk, namely those which can actually be transmitted to the cow and result in the profuse discharge of the infecting agent in its milk. This group III which includes especially the streptococcal infections, scarlet fever and epidemic tonsillitis, though in bacteriology it is for the most part identical with II, requires differentiation because of the intensity of the milk infection and the widespread nature of the resulting epidemics. It was, in fact, the exaltation of the infecting power of milk observed in certain epidemics which led to the investigation and proof of this type of infection, since it seemed incredible that droplet infection or hand or utensil contamination of milk after discharge from the cow could introduce sufficient infective material to convey disease to so high a proportion of consumers of a milk diluted by bulking a large number of different supplies.

As regards the effect of milk-borne infection generally on the public health, instructive figures have been collected for the United States by Crumbine(28) and by Borman *et al.*(29). The former gives the following table for eighteen of the States for the year 1934.

Disease	Epidemics	Cases	Deaths
Diphtheria	1	9	0
Gastro-enteritis	4	243	0
Scarlet fever	3	128	2
Septic sore throat	9	610	15
Typhoid fever	21	315	25
Undulant fever	2	19	2
Total	40	1324	44

The latter give the statistics of 760 outbreaks ascribed to milk in the U.S.A. for the years 1910–33.

Disease	Outbreaks	Known cases	Deaths
Typhoid fever	574	8459	406
Septic sore throat	81	26773	226
Scarlet fever	72	5326	40
Diphtheria	33	1083	11

Frank(30) states that, on the average in the U.S.A., forty-three milk-borne outbreaks of disease occurred annually from 1924 to 1933, not including those of gastro-enteritis.

For Great Britain Wilson(31) gives the following figures for 1912–31 and is careful to point out that they probably represent but a fraction of the total number of outbreaks that have occurred as the result of ingesting infected milk.

Disease	Outbreaks	Persons affected
Scarlet fever and septic sore throat	25	3087
Diphtheria	12	732
Typhoid fever, paratyphoid fever and dysentery	22	1843
Gastro-enteritis	10	3759
Total	69	9421

(These figures do not include twelve other outbreaks in which the number of persons affected is not obtainable.)

It will be observed that tuberculosis of bovine origin does not appear in any of these tables since it rarely occurs in recognizable epidemics. Its total incidence is, at least, 5000 cases annually in Great Britain (Wilson(31)). The incidence of undulant fever, much of which is certainly of milk-borne origin, is similarly hard to estimate. Wilson(31) suggests that there may be some 500 cases annually in Great Britain and this is likely to be an under-estimate rather than an exaggeration. Olin(32) estimates the incidence in Germany at 8·3 cases, in Sweden 18·4 and in Denmark 150 per million of the population. Stone & Bogen(33) made observations on 854 persons known to have consumed milk heavily infected with *Br. abortus*; sixty-eight showed specific agglutinin in their blood but only half of these had any suggestive clinical symptoms and only three an illness recognizable as mild undulant fever.

There is much that is still mysterious about the infectivity for man of *Br. abortus* ingested in milk. The abnormal incidence of undulant fever in all countries on males (about $2\frac{1}{2}$ times that on females) among adults, whereas among children the sexes are equally affected, suggests that the regular ingestion of subinfective doses of the specific bacteria may prevent the abortus septicaemia which constitutes the clinical disease. It is to be remembered, too, that the brucellas, to judge from the number of subspecies recognizable by biological peculiarities and associated with particular regional distribution, appear to be a bacterial genus undergoing active evolution and that this evolution may take the direction of abnormal pathogenicity for man under conditions which can only be guessed at.

This special incidence of milk-borne epidemic diseases on women and children, as greater consumers of milk than adult men, has been discussed by Picken(34) and by Hill & Mitra(35). The former points out that the statistics in America and Scotland for the throat and intestinal infections do not support it, while the latter, on comparing twenty-nine water-borne with thirty-one milk-borne outbreaks, chiefly of enteric fever, find that there is, in general, though with many exceptions, such a special incidence. In the case of scarlet fever it must be remembered that the statistics may be falsified by the relative immunity of adults to the rash-producing toxin so that they escape notice as cases of the disease, though in fact infected and reacting with throat inflammation and its common sequelae.

There is no doubt that human infection with the bovine tubercle bacillus, largely alimentary, is much commer in children than in adults and may justly be regarded as an example of special incidence of milk-borne disease; here again, however, a relative adult immunity may partly explain the exaggerated puerile incidence. The conclusion must be that, though the statistics of age and sex distribution may confirm a hypothesis of milk-borne infection, absence of such confirmation, as for example in the case of undulant fever, does not invalidate it.

As indicated above, epidemics of the suggested group III, i.e. those in which the cow acquires, harbours and discharges in its milk a normally human bacterial parasite, furnish the most striking example of milk-borne outbreaks, the great majority being streptococcal. Some discussion is necessary of the bacteriological data which have permitted this conclusion. The association of streptococcal infection of the udders of cows with widespread outbreaks of scarlet fever or non-eruptive

streptococcal tonsillitis among consumers of their milk was observed by Klein in the early days (1885) of bacteriology. The difficulty, however, of distinguishing with certainly the streptococci of the septic throat from those found quite commonly in ordinary milk and the fact that streptococcal infection of the udder, with acute mastitis, need not render the milk pathogenic for man, robbed the observations of much of their significance. It is only in quite recent years that conclusive arguments have been brought, chiefly by serological methods, to identify the human pathogen in the cow. Evans(36) gives a list of American epidemics since 1927 in which the *Streptococcus* concerned was found also in the cow and discusses the proofs of identity. These are, in order of precision, the production of similar haemolysin and toxin, the same group carbohydrate antigen (group A), as revealed by the precipitin test, and the type agglutination (by Griffith's drop method).

An epidemic of scarlet fever in Doncaster towards the end of 1936, details of which have been published by Watson(37), has furnished an excellent British example. The infection was traced to a particular cow not suffering from clinical mastitis but discharging in its milk enormous numbers of streptococci of the same precipitin group (A) and agglutinin type (2) as those found in the throats of the human sufferers; this cow developed later subacute mastitis in one affected quarter and continued for weeks to harbour in it the same *Streptococcus*.

The source of infection of the cow in such instances is, of course, human but the actual method of inoculation of the udder has been the subject of considerable debate. Experimentally, it has not proved easy to induce infection of the teat canal and mammary gland by simple application or scarification of the surface of the teat with streptococcal culture. Injection into the teat canal succeeds but such inoculation can bear little relation to the "natural" method. Rambe & Hedström(38) and Christiansen & Nielsen(39) offer the most likely explanation; it is that ulceration or even abnormal dilatation, e.g. by sphincter paralysis, of the actual orifice reduces or destroys the natural defensive mechanism of the teat canal.

Group III, as defined above, though made up chiefly of milk-borne epidemics of throat infection, may not be limited to these. There is the possibility that such specifically human diseases as typhoid and paratyphoid fever may on rare occasions become infections of the cow. No convincing facts to bear this out are at present available but certain epidemiological observations suggest that it is well worth experimental test.

Prophylactic measures against milk-borne infection hardly require detailed discussion. It is not too much to say that there is no near prospect of raw milk becoming a safe, disease-free food; proper pasteurization, or in default of that, boiling, of all milk should be enjoined by all hygienists in all countries. The subsequent handling of such milk should, of course, be attended with even greater precautions than that of other foods to prevent the introduction of infective material of human origin. But post-pasteurization infection has so far been only rarely the cause of disease, in spite of the obvious opportunities furnished too often by present methods. The *Salmonella* infections, including typhoid and paratyphoid, which may be introduced by temporary or chronic "carriers" should be guarded against by health supervision of dairy employees and by reducing hand contact to a minimum. Droplet infection is, perhaps, less easy to prevent, though, as in other food preparation processes, the wearing of proper face masks, if it could be enforced upon the workers, would reduce it to a minimum. Other methods of preventing it, such as regular "swabbing" of throat and nose of milk handlers, have little prospect of success. Foote *et al.* (40) for example, tried weekly swabbing of eighty-five dairy employees (throat and nose): they found that by the end of 3 months 20 (= 23 per cent) had on at least one occasion given a positive swab (streptococci of group A), though there was no

evidence that their "carrier" state had infected the milk or indeed was capable of conveying disease even directly.

Such a routine is, in fact, like that of trying to prevent puerperal infection by frequent swabbing of obstetrical attendants and is equally impracticable.

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